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STUDIES IN BIOPHYSICS:

The Critical Temperature of Serum (56°)

By

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TO MY WIFE

who performed a large number of the measurements reported in this book, and whose untiring and skillful activity in the course of so many years has been a comfort and an inspiration to me.

P. L. N.

Foreword

The experimental researches condensed in this book by no means cover the whole subject. However they constitute a systematic effort to pave the way so that it will eventually be possible to tackle the still mysterious problems of immunity by means of physico-chemical and physical methods. The epoch-making work of the late Karl Landsteiner, of Oswald Avery and of their collaborators, has opened the door to a chemical approach of the subject. So far, little has been done in the field of physical chemistry, which remains practically unexplored.

A certain number of new facts will be described. They throw some light on the fundamental problems relating to the mechanism of immunological reactions. They have transported these problems into a different region of science, where accurate measurements are possible. They have disclosed new problems, as yet unsolved. They have demonstrated that a systematic study of the serum, based on sound working hypotheses, was capable of revealing an important number of unknown phenomena. Each of these new facts can be considered as a starting point for other researches in the same direction, or in a direction more specifically chemical. The author does not claim that he has supplied all the answers, but hopes that he has succeeded in demonstrating that, by refining old techniques, or creating new ones and by confronting the results critically, it is possible to open up unsuspected fields of research.

All biological and medical problems involve phenomena of a physico-chemical nature which, until now, have often been neglected. The number of facts unearthed in the past twenty years has been so great that there has been no time to study them thoroughly one by one. Laboratories were not only short of time, but of men. These problems, as soon as they were stated, should have been taken over by pure physico-chemists. Had this been done, the ground would have been progressively consolidated through this ideal cooperation, without which continuous progress is impossible.

We are faced today by an enormous mass of observations, more or less accurate, piled up in disorder and frequently disconnected. It will take a long time to establish a relation between all these elements, but the longer we wait, the harder the task will be. In order to accomplish this, it seems that some kind of centralizing organization would be required, similar to the clearing-houses in the world of finance, where sorting and tentative

classification could be done. But the distribution of the work would undoubtedly raise new difficulties, hard to overcome.[1]

For want of a broadly conceived and comprehensive plan, and for lack of a material organization aimed at the purely scientific approach to these problems, the basic mechanisms of immunity, and of many other questions, will remain obscure for a long time to come, not so much on account of our ignorance as on account of the dispersion of competent efforts, of the absence of coördination and of efficient cooperation between the various scientific disciplines.

The writer has attempted to interpret some of the new facts brought to light by means of a hypothesis concerning the structure and the behavior of the complex lipido-proteinic "molecule" of serum. He is aware of the fact that this conception does not always tally with the ideas propounded by other specialists. As these theories were evolved before the new phenomena were disclosed, he feels that he was entitled to propose, tentatively, a working hypothesis which seems to explain satisfactorily the behavior of serum as a function of temperature. This hypothesis proved to be useful in planning further experiments which resulted in the discovery of more unknown phenomena. It has, therefore, been satisfactory and constructive, and this is, momentarily, the only claim which the writer wishes to make in its behalf.

LECOMTE DU NOÜY

New York, N. Y.
April, 1945

[1] A very interesting paper was recently published in "Science" by Dr. Joseph Brozek and Professor Ancel Keys (December 8, 1944), under the title "*General aspect of interdisciplinary research in experimental human biology.*" Although dealing with the specific problem of human biology, the authors insist on the necessity of what they aptly term "*interdisciplinary research.*"

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Chapter 1

Introduction

Blood collected from the arteries or veins of a living animal coagulates into a gel. This coagulum with a fibrin base, contracts, imprisons the red and white cells and exudes a yellow liquid which constitutes the serum. As the fibrin remains in the clot, the serum admittedly represents the total blood, minus the fibrin and figured elements. Plasma is differentiated from serum because it contains fibrin, or more exactly fibrinogen which is transformed into fibrin during coagulation.

Numerous works have been published on the physical and physico-chemical properties of serum. About 1,321 papers and works are cited in the Brocq-Rousseu and Roussel[1] bibliography which is now ten years old. The chemical papers can be counted by the thousands.

These works, with few exceptions, do not lead to important results outside of the statistical information obtained and the doubtful interest of possessing an immense amount of numerical data corresponding to the physical properties of a biological liquid, the so-called "normal" serum which, in reality, is non-existent. Until 1923, only a small number of workers, among whom were Ascoli, Izara, Zunz and Vlès, conducted systematic experiments crowned with success, but in which the properties of serum were considered more as the expression of a physiological or pathological state than as a distinct problem.

In 1920, we conceived the project of studying serum as a complex, fragile liquid, by avoiding all brutal manipulations capable of destroying the properties which we were trying to define. We decided to examine carefully the mechanisms of certain phenomena and thus try to throw some light on the mysterious, fundamental reactions which are so precious in medicine, particularly for the diagnosis of infectious diseases.

To accomplish this it was necessary to accept the classical definition of "normal" serum: the serum of a healthy animal having received no immunizing injection. But it was also necessary to eliminate innumerable factors, known and unknown, which quantitatively influence all the measure-

[1] "Le Serum Normal," Paris, Masson, 1934.

ments. A general method which had one great advantage and a slight disadvantage imposed itself. This method consists in always studying a serum "kinematically" and not statically, that is, in replacing the idea of *mean value* (represented by a point in Cartesian coördinates) by the idea of the variations of this value as a function of a known factor, these variations being expressed by a curve.

For instance, instead of measuring the surface tension of a hundred sera of a given species of normal animal, then adding all these values and dividing the total by 100, in order to obtain the arithmetical mean, it was proposed to study the variations of surface tension as a function of time, of dilution, or of temperature, while maintaining all the other factors constant. If the phenomenon is studied as a function of time, the dilution and temperature are kept constant. A second series of measurements will be made in the same way, but starting with a different dilution. All the dilutions will be studied successively as a function of time, at a constant temperature, so that it will be possible to deduce the influence of the dilution and of the temperature from a comparison of all the curves obtained. The same series of experiments is then repeated, the dilution being kept constant and the temperature varied. Finally, the characteristics of this particular serum will be known for each dilution and each temperature as a function of time. As time cannot be immobilized, it is obligatory, when wishing to eliminate it, to act quickly and to assume that the influence of time becomes negligible, or else to consider it as an invariant in each experiment. Its influence, being the same, will then affect the phenomenon as a whole, by displacing the entire curve, but without altering its shape, which is the object of the experiment.

If all the sera are subjected to the same series of experiments, the curves will be higher or lower with reference to the axis of ordinates, according to each particular sample; but the *shape*, as a function of the variable, will be approximately the same. If there is an important individual deviation, it is an indication of an unquestionable anomaly which could have escaped attention in the case of a single measurement, but which has far greater chances of manifesting itself when the conditions are progressively and systematically varied.

Finally, as each curve is the result of experiments on the same serum, the result is homogeneous and has a definite meaning. The same cannot be said of a figure expressing the mean value of certain properties pertaining to a number of different sera. For instance, the influence of immunization on the surface tension, which cannot be shown by measurements on pure serum, becomes more and more marked when the serum is progressively diluted. It attains a maximum value at a certain critical dilution (1/11,000

for rabbit serum), all other conditions being equal. This phenomenon had remained unknown until we applied the method in question.[2]

Another example: the "mean" specific viscosity of horse serum has been determined by numerous workers. One of them gives the value of 1.6 at 20°; another 1.7; a third 1.8; a fourth 1.55. The "mean of mean values" is 1.66. This value has no significance. The second decimal point is absolutely illusory. The mean values differ because the horses were not of the same race, were fed differently, were bled at different hours, and above all because the precision of the methods employed left much to be desired. If, on the contrary, the *variations* of the viscosity of horse serum are studied as a function of time, in a continuous fashion, one observes that there is an absolute minimum of viscosity at 56° and that the latter increases rapidly above this temperature. This has more significance, for we know, on the other hand, that 56° is the temperature which corresponds to the "destruction of the complement," an important biological property which is thus found to be related to a well defined physical phenomenon.

It was also found that the serum of a great number of animals belonging to different species, and that of man, possessed the same characteristic, and that this is not true for current colloidal solutions: gelatin, gum arabic, etc. The kinematic (some would call it dynamic) method thus revealed a general phenomenon characterizing blood serum.

To follow the principle of the method it was also necessary to study the influence of the duration of heating. The results, which we shall examine later, were equally interesting and unforeseen. The method is therefore very general, as it is possible in certain cases to efface not only individual differences but also differences of species.

The immediate objections that can be made are the following: the application of this method demands an enormous number of measurements and a considerable accumulation of material. Each measurement, if it is carefully done, takes time. A life-time would not suffice. What is more, as each curve should be the result of experiments made with the same serum, great quantities of blood are needed. This is not practicable if one is working on small animals.

This is all true, or rather was true when we started this work (1920). We shall try to show how it is possible to get around the difficulty. It is evidently necessary to amass a great number of experiments. This is true of almost all biological work. Consequently, one should be able to accumulate measurements performed by non-specialized assistants; these must be made rapidly without a corresponding decrease in the high accuracy

[2] Lecomte du Noüy, *J. Exp. Med.*, 37, 659 (1923); 40, 129 (1924); 41, 779 (1925) etc., and in "Surface Equilibria of Biological and Organic Colloids, New York, Reinhold Publishing Corp., 1926.

required. The improvement of all techniques is, therefore, necessary so that these requirements may be met. If it takes thirty seconds instead of one hour to make a measurement of surface tension, 374 measurements a day can be made, as was the case in our laboratory, including all the necessary manipulations. This naturally calls for special equipment, organization, and rationalization of the work. The ideal equipment must, therefore, be conceived with the object of performing rapid measurements without loss of precision, and of permitting large series of measurements. In all cases it is more advantageous, as we have frequently experienced, to spend several months realizing and perfecting a new instrument and a technique exactly adapted to the problem, than to attack the question with inadequate equipment. The "lost" time is quickly recovered; and, in any case, there remains a perfected technique which may be useful to others.

Moreover, it is evident that all the working hours of a day or week are rarely spent in perfecting a new method. There are always other experiments going on, and the extra effort, besides being in general fascinating, is usually rewarded by the results obtained.

Our first experiments on viscosity were made with Ostwald's instrument, in a large thermostat, and required about one hour for each measurement. As we had to make one measurement for each degree between 20° and 70°, it took us about 50 hours, or ten hours a day for five days. We needed 500 cc of serum. When taken from the same animal, it probably underwent changes at the end of this lapse of time; and only a large animal, *i.e.*, horse or ox, could supply the required quantity. For rabbits, chickens, or guinea pigs, several animals a day were needed. When our new equipment was ready we could make about 100 measurements in $1\frac{1}{2}$ hours (from 20° to 70° and from 70° to 20° by cooling) with 1 cc of liquid. The instrument was sensitive to the 5th decimal point (c.g.s. units). No manipulation was necessary: the worker, sitting down, simply inscribed the figures read on the scale, unless they were automatically recorded. We were thus able to make 50,000 measurements on all kinds of liquids in a few years without interrupting the other work of the laboratory. It took about six months to design, construct and perfect the new viscosimeter.

We have purposely dwelt on these preliminary details so as to make it clear to the reader that *the necessary foundation of the method which we employed* was the creation of new instruments particularly adapted to biological solutions, or the modification of old instruments with the same object in view, and the perfecting of special techniques, simple enough to be used by any assistant and destined to save time. In brief, what we did was to apply to the laboratory the principles used currently in industry since the beginning of the century. The reason for the standardization, rationalization and Taylorization in factories is to decrease the cost by mass

production, based on the use of high-precision calibers. Our aim was to increase the number of measurements and their precision, by eliminating as far as possible either the individual factor or the manipulations in which this factor could intervene. This led us sometimes to simplify, but more generally to complicate the instruments. Every complication, however, was motivated by a simplification in its handling and a greater guarantee of precision in the measurements.

Usually, when it is necessary to multiply measurements of a certain nature, it is not the measurement itself which takes time but the manipulations preceding or following it: cleaning and sterilization of the vessels containing the liquid, preparation of accurately titrated solutions, decantation into special vessels, and unavoidable lapse of time to obtain the equilibrium (temperature, adsorption, potential, etc.). Moreover, after each measurement, the apparatus must be put in order for a second measurement. In certain cases the measurement takes half a minute or less, but preparation of all the essential elements takes a whole day, or even more. It is, therefore, indispensable that the work be *organized* in such a way that the accessory details do not have to be repeated for each measurement, but that on the contrary they can be prepared in view of a large number of identical measurements. This necessarily entails a rationalization which greatly resembles the methods of industry, and which saves much time without affecting the precision.

The only objection that can be raised to this system is that it requires more glassware, apparatus, and room, and consequently, more money. But besides the fact that this is not always true, it cannot be called an objection. It is a condemnation of the organizations and men in charge of the destiny of scientific culture. Everything depends on the object in view. If one is satisfied with old-fashioned laboratories, in which work is slow and difficult, without comprehending the part played by pure research in general culture and intellectual prestige, and without being convinced of its ever-increasing economic value, then one always spends too much money, inasmuch as this money is not employed to its maximum value. If, on the other hand, one is honestly determined to make human knowledge progress, because one believes that only through science will scourges, such as infectious diseases and cancer, be finally controlled, then one can never spend enough money. Everything, in brief, depends on the value attributed to human life, health, and the progress of knowledge.

When we undertook the systematic study of the physical and physicochemical properties of blood serum to try to throw some light on the structure of this complex and on the real mechanisms of immunity reactions,

we were fully aware that the task was a considerable one and we were convinced that a life-time would not suffice.

More than twenty years have passed. The facts discovered have proved that we were right in thinking that a great part of the obscurity surrounding these phenomena was due to our ignorance of the fundamental facts and to the errors of conception thus involved. However, they have also brought the conviction that the work accomplished was negligible in comparison with what remains to be done, and that all efforts would be futile until the chemistry of fragile bodies, four-dimensional chemistry—in other words, the chemistry of proteins—had progressed.

The two expressions "chemistry of fragile bodies" and "four-dimensional chemistry," can be considered synonymous in the case of proteins. Indeed, if we agree to call "fragile body" a body the properties of which are not stable as a function of time and vary, often irreversibly, from one moment to another, we are forced to introduce time as a fourth dimension in our very definitions. Time has already had to be introduced into the chemistry of radioactive bodies which are perpetually transformed into one another before they reach a stable ultimate state, such as lead in the uranium family. But the problem is infinitely more complex in the case of proteins comprising thousands of atoms, with molecular weights varying between 16,000 and 6,600,000, and which are not chemically defined at any time.

Here we must point out that when we speak of the fragility of proteins, we mean the fragility of the entire structure, and not that of the constitutive elements which are chemically determined groups and, on the contrary, possess great stability. Similarly, when a child builds a house out of solid blocks, the fragility of the edifice increases in direct proportion to its complication. The physico-chemical and biological properties of a protein sometimes depend on the stable groups, but also frequently on their combinations, which are more and more unstable as the number of combined groups increases. Two kinds of properties can, therefore, be roughly distinguished in a protein molecule: (1) the chemical properties due to the nature of certain stable elements which compose it, (specificity among others), and (2) less well defined physico-chemical and biological properties which emerge from the stereochemical *coupling* of a more or less large number of stable chemical groups (antigenic property ?).

The chemical groups which determine the specificity, for example, do not react in a simple way. Their reactions depend on the chemical bonds which link them to other much more complex groups endowed with antigenic properties lacking in the first ones. The first (haptenes) only serve to orient specifically the reactions which would be impossible without the presence of the second group and without the combination of these two elements. At least, this is the way in which these phenomena can be actually conceived.

New properties and a new fragility are born from the combination itself.

The substance of our experiments on serum was condensed in about thirty papers and one book. Two different kinds of results stand out when the work is considered as a whole. First, the new material results, purely experimental, which constitute a group of independent phenomena easily reproducible, and which were often checked, but were apparently unrelated, except in a few cases. Secondly, the conclusions which we derived from them. Our logical interpretation, often based on quantitative relations, is, as was stated before, definitely hypothetical; but it acquires an important character of probability from the fact that the principal hypothesis enables one to establish a coherent link between all the observed facts, which thus derive logically one from another. In other words, this hypothesis, which proved fruitful inasmuch as it suggested numerous experiments, transforms a succession of points into a regular curve. Each phenomenon loses its independence with respect to the others. It becomes one of the aspects of the fundamental phenomenon and its individuality is engulfed in that of the curve which represents the evolution as a whole. This hypothesis was very useful as a working tool. From every point of view it seems to be more plausible and efficacious than the classical hypothesis which it replaces and which, as we shall show, is incapable of explaining and coördinating the new facts.

The classical hypothesis is that of colloidal serum. The new hypothesis is that of a serum molecularly dispersed, comprising albumin, globulins, lipoids, etc., in a true solution, either in a state of more or less fragile combinations, or free. For a long time we thought that the majority of proteins existed in the shape of a large "serum molecule." The latest experiments made in Svedberg's laboratory indicate, on the contrary, that though the dispersion is molecular, as we assumed, there are four distinct groups of molecules, two of which are far more numerous than the others.[3]

[3] Mutzenbecher, P. v., *Biochem. Z.*, 266, 226 (1933). I will give a short summary of the principal results obtained.

The experiments made by the author, by means of the ultracentrifugation method, led him to the conclusion that horse and human sera contained four different molecules in varying proportions with sedimentation constants of 4.5, 6.8.9,17, ($\times 10^{-13}$). The two first, which are the most numerous, correspond to the albumins and globulins of serum; the last two correspond to larger molecules which are present only in small quantity.

The writer has shown that molecules twice as small as the albumins are present in old, undiluted serum. It was possible to prove that, in a serum diluted in isotonic solution, still smaller molecules exist which increase in number with time. These elements are probably the result of cleavages.

The globulin-albumin ratio also changes with the dilution, the relative proportion of globulins being increased. This apparent increase is perhaps due to the fact that, in undiluted serum, the globulins form complex associations the number of which, however, is too limited to make it possible for them to be observed by ultracentrifugation. It is possible, as I wrote in 1930, [Revue Scientifique, Feb. 8, 1930, p. 66,] that an equilibrium

In the first hypothesis the properties of the substances constituting the serum would be in part due to their state of aggregation in the shape of micelles. In the second, these same properties would be the result of their valences and of their chemical structure in space, as well as of their physical and physico-chemical properties, which are the consequences of their nature and of their molecular dimensions. At no time, under normal conditions, would aggregation take place. The proteins—or rather the complex, lipoprotein, of which we have spoken—are and remain molecularly dispersed, as a true solution.

The first hypothesis closes the door to a chemical interpretation of the specificity of serum or plasma. The second, on the contrary, opens up vast horizons to this specificity and makes it possible to introduce into biology the rich and fertile idea of monomolecular oriented dispersion, which we have upheld since 1923.

As our experimental work dealing with oriented adsorption has already been developed in a preceding book,[4] we will limit ourselves to summarizing it briefly. On the contrary, we shall develop in detail all our subsequent work which was mainly done at the Pasteur Institute from 1927 to 1937, and which is closely related to the fundamental problems on the nature of alexin, of the sensitizer, and of the mechanisms of the action of the complement.

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exists between the large and more complex molecules and their smaller elements, and that the mean properties of serum correspond to the top of a Gauss probability curve.

Biochem. Z., 266, 250 (1933). The globulins of serum obtained by precipitation with semi-saturated ammonium sulfate are polydispersed. The greater part are formed by molecules having a molecular weight of about 138,000.

The serum albumin obtained by dialysis of the "Gesamt albumine" is almost monodispersed (there is a small quantity of smaller molecules). Serum albumin precipitated by saturated ammonium sulfate contains the product of association of several molecules.

In all the albumin preparations it was found that the principal molecules had a constant of sedimentation of 4.5×10^{-13} (corresponding to $P_m = 69{,}000$).

Biochem. Z., 266, 259 (1933). If increasing quantities of ammonium sulfate are added to the serum, small molecules appear and their quantity increases with the concentration of sulfate.

Electrodialysis entails a partial association of proteins which can be reversible or irreversible.

[4] Lecomte du Noüy, "Surface Equilibria of Biological and Organic Colloids, New York, Reinhold Publishing Corp., 1926, and "Equilibres Superficiels des Solutions Colloidales" *Monographie de l'Institut Pasteur*, Paris, Masson, 1929.

Chapter 2

Adsorption; Monomolecular Layers; Summary of Previous Experiments

As reported above, all our work on serum can be divided into two distinct groups: the first leads to the concept of polar "serum molecules," adsorbable in monomolecular layers at the interfaces. The second leads to much greater precision concerning these molecules, which are shown to be asymmetrical, anisotropic, capable of being irreversibly hydrated by fixation of intramolecular water, and of swelling individually when heated, until a gel is formed.

The first group explains the difference between *in vivo* and *in vitro* reactions by introducing the idea of catalysis through surface action in the cells and organs; the second studies coagulation by heat, the physico-chemical nature of the destruction of "alexin" by heating at 56°, and shows the difference between this phenomenon and that of the destruction of the "sensitizer" at 66°.

Even if the reader is not interested in our hypothesis, or does not approve our interpretation of the phenomena, we hope that the following account of our experiments and of the new facts observed will give him the necessary elements to enlighten his own point of view and to enable him to build up a new theory which will satisfy him more completely.

The fundamental phenomenon of the first group is the absolute minimum of surface tension of serum diluted in a physiological solution. The accessory phenomena are:

(A) The shift of the minimum of surface tension as a function of the ratio S/V (S = surface of adsorption and V = the volume of the solution).

(B) The anomalies in the rate of evaporation of serum solutions.

(C) The aspect of the NaCl crystals of the solution after evaporation at the critical concentration corresponding to a given S/V ratio.

We shall rapidly remind the reader of the nature of these phenomena, which were described previously.[1]

[1] Lecomte du Noüy, "Surface Equilibria of Biological and Organic Colloids," New York, Reinhold Publishing Corp., 1926.

Minimum and Shift of the Minimum

Rabbit serum diluted in an isotonic solution shows an absolute minimum of surface tension when the static value is sought, that is, when the measurements are made after the adsorption equilibrium has been established. This absolute minimum is found at a dilution of about 1/10,000th when the measurements are made in vessels in which the ratio S/V is equal to 13.2 (calibrated watch-glasses and 2 cc of solution). Under these conditions the thickness of the adsorbed protein layer is approximately 4 m μ, or 40 Angstroms. When the value of the ratio S/V is changed, in other words when the surface is increased or decreased and the volume (*i.e.*, the number of molecules) is kept constant, the mean thickness of the adsorbed layer, or more exactly the number of molecules adsorbed per unit of surface, varies and brings about a variation in surface tension. This surface tension shows a minimum when the adsorbed layer is strictly monomolecular and without solution of continuity, that is to say, when it forms a homogeneous film. If the concentration and the ratio S/V are varied simultaneously, and if the same surface of adsorption is available to the same number of molecules, the place of the minimum observed under the conditions defined above can be shifted at will, and occurs at any chosen concentration. This was established by experiments made with protein molecules and confirmed with sodium oleate molecules.[2] The phenomenon would be incomprehensible if each molecule did not always occupy the same space, *i.e.*, if it were not capable of orienting itself in a monomolecular layer, geometrically organized, like identical prisms of cork pressed one against the other and floating on water.[3]

Anomalies in the Evaporation Rate of Diluted Serum Solutions

When solutions of sera are left in watch-glasses having an S/V ratio equal to 13.2, at room temperature, it is found that the solution at 1/10,000 evaporates more slowly than others at 1/10, 1/100, 1/1,000, and 1/100,000. The explanation of this phenomenon which we proposed rests on the same hypothesis as the preceding one, namely the existence, at that concentration, of an oriented monolayer which impedes the escape of the molecules of water by its solid, homogeneous structure. This would tend to prove that the

[2] The existence of 3 minima and of their shift, observed in sodium oleate solutions, first reported by the writer in 1924, [*Phil. Mag.*, 48, 664-672; *J. de Phys. et Radium*, 6, ser. 6, 145-153, (1925); "Surface Equilibria," chap. 4, p. 86, (1926)], have been checked by different authors; the most recent check was published in *Compt. Rend. Ac. Sc.* in 1940 by Dr. Herquet (University of Liège, Belgium, paper presented by M. de Broglie). Unfortunately, during the latter part of 1940 this journal never reached the U. S. on account of the war, and the writer regrets that he is unable to supply the exact bibliographical information.

[3] Lecomte du Noüy, *op. cit.*, Reinhold Publishing Corp., pp. 78-80 and 86-89.

organization of the adsorbed layer is perfect only when it is unique and is not superposed on other subjacent layers.[4]

Aspect of NaCl Crystals after Evaporation

The aspect of these crystals is totally different, in the case of immunized serum diluted to 1/10,500, from that of the crystals grown from more or less concentrated solutions. At this critical concentration (the value of the ratio S/V being always equal to 13.2) crystals shaped like fish-bones can be observed, which indicates the presence of forces capable of orienting the crystalloidal molecules, while in normal serum, under identical conditions, the crystals show no such patterns.[5] A similar phenomenon takes place with sodium oleate in presence of NaCl, evaporated under such conditions that a monomolecular layer is formed (1/750,000). The explanation of this phenomenon can be found only in an oriented state of adsorbed molecules.

It is quite evident that the hypothesis of a truly colloidal solution of proteins, *i.e.*, isotropic non-polar micelles, incapable of being oriented or of imposing an orientation on the crystalloidal molecules present, cannot explain any of the above phenomena. Neither can it explain any of the remarkable phenomena (hemi-wettability among others) discovered by Devaux, who studied other proteins. On the other hand, the hypothesis of molecules in true solution explains, and in certain cases (displacement of the minimum) enables one to calculate and to foresee the facts quantitatively when the conditions of the experiments are systematically altered. We shall see later on the important consequences that can be deduced from this.

We may add that the calculated thickness of the monolayer of serum proteins is in satisfactory accord with that calculated by Fricke for the thickness of the red blood-corpuscle "membrane," namely 33 instead of 35 Angstroms (3.3 m μ instead of 3.5 m μ).

[4] This phenomenon was first described in 1924 (Lecomte du Noüy, *J. Exp. Med.*, **39**, 717). It was rediscovered in 1925 by Rideal, and extensively investigated by I. Langmuir and V. Schaefer, (with different chemicals). See: "Surface Chemistry," 17, (Publ. of Am. Assn. Adv. Sci., No. 21, 1943).

[5] Lecomte du Noüy, *op. cit.*, Reinhold Publishing Corp., pp. 142-143.

Chapter 3

The Viscosity of Serum as a Function of Temperature

The viscosity of colloidal solutions is not easily determined by the methods which involve the use of a glass capillary. The technical difficulties encountered are important, and when successfully surmounted they still require a relatively large amount of liquid. Moreover, they do not permit the continuous observation of the variations which may take place in a solution as a consequence of a reaction, or under the influence of temperature or of time. These were the main reasons which led us to establish a viscosimeter which would escape the above limitations.

The original instrument used in the following experiments was described a long time ago,[1] but since then it has been considerably improved.[2] It is fundamentally based on the principle of two coaxial cylinders; the outside cylinder, or cup, which contains about 1 cc of liquid, rotates at a constant speed, and the inside cylinder, or bob, is suspended by means of a galvanometer wire. In the new model the sensitivity was not increased, as the instrument was already too sensitive if anything; suffice it to say that the first model made it possible to obtain readings to the sixth decimal point in c.g.s. values (0.000,001), so that the viscosity of air could be used to standardize the instrument (0.000,185,8 at 25°).

The viscosity of water thus determined, at the same temperature, yielded a value of $\eta = 0.008,916$, in excellent accord with the value given by Thorpe and Rodger (1894) of 0.008,91. The sixth decimal can be neglected, as a difference of 1/100th of a degree, between 20° and 30°, introduces an error of 2 units in the 5th decimal place. An experimental study of errors showed that, under the most exacting conditions, a relative error of 0.025 per cent was to be expected. Practically, an error of 0.1 per cent was considered as acceptable. As a difference of 0.01° in the temperature introduces an error of the order of 0.2 per cent, this indicates that an overall accuracy of ± 0.3 per cent is the best that can be expected under normal working conditions, when the temperature cannot be relied upon to more than 1/100th of a degree.

It was soon found that the significant phenomena were of such an order

[1] Lecomte du Noüy, *Jour. Gen. Physiol.*, **5**, 429 (1923).

[2] It is on the market in Europe, but not in the United States.

of magnitude that this sensitivity was unnecessary, and that the time element was of greater importance. Therefore, we modified the original instrument so as to increase its range and to make readings easier.

This was done in the following way. The phosphor-bronze suspension of the plunger, or bob, is used to carry a feeble current through a d'Arsonval coil moving between the poles of a horseshoe magnet. Under

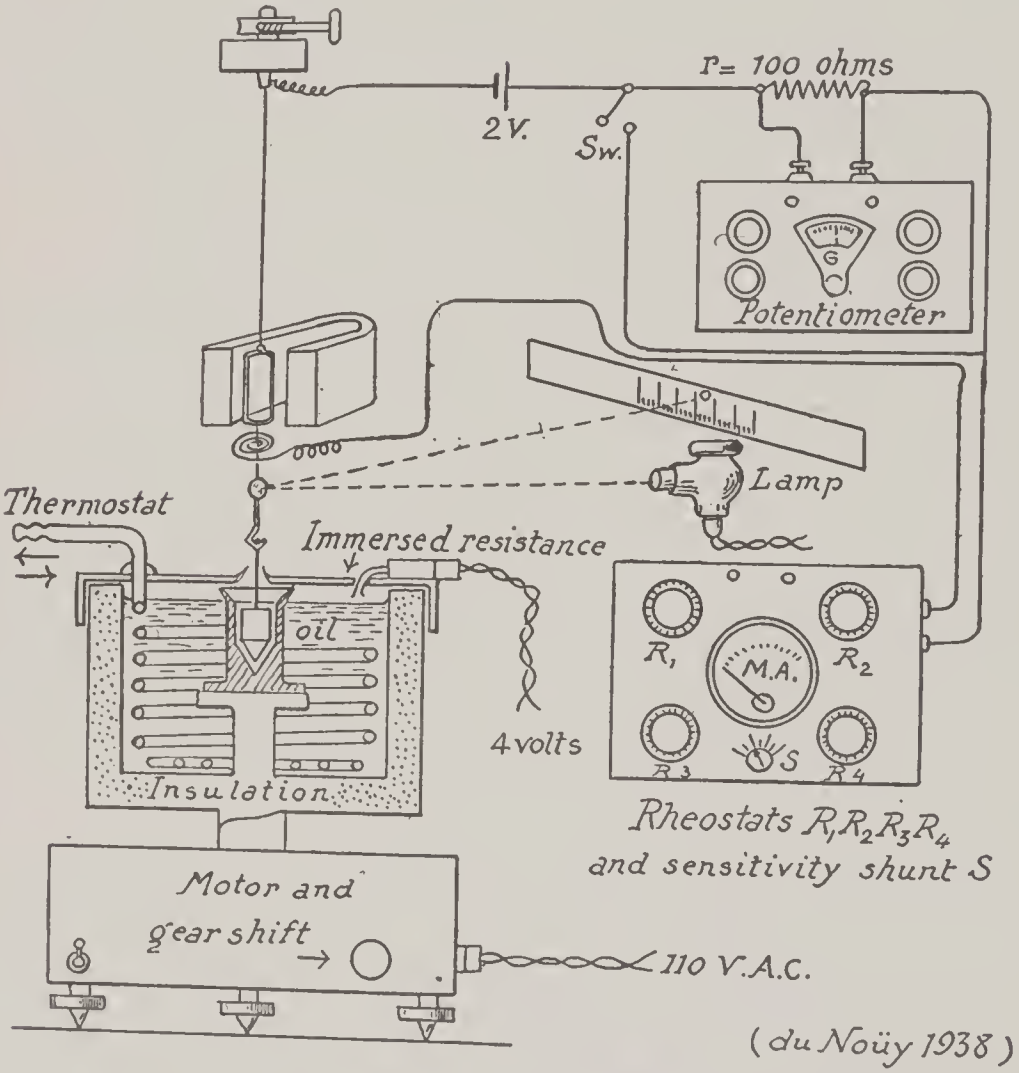

Fig. 1. Schematic assembly of the du Noüy Viscosimeter.

this coil is the mirror, and the hook to which the bob is suspended. The suspension is non-conductive of heat. The current flowing through the coil is led out through a thin spiral above the mirror (Fig. 1), just as in an ordinary galvanometer. The current from one or two dry cells passes through a milliammeter and a series of four variable rheostats, so that it can be very accurately and progressively adjusted, the first one giving the coarser and the fourth the finer adjustment. For accurate measurements (to the fifth significant figure) a special set-up can be thrown in by a switch which is normally short-circuited.

In order to make an experiment (*i.e.*, a relative determination of viscosity as a function of time and temperature) the following procedure is followed. The position of the zero having been checked and found perfectly stable, the constant-speed motor driving the cup containing the serum (1 or 2 cc, according to the size of the cup and bob) is started; through reducing gears it imparts to the cup a speed which can be varied from 2 to 40 rpm. (The speed generally used was 16 rpm). At once the spot begins to move. Simultaneously, the observer, by acting on the dial controlling the first rheostat (coarse adjustment), sends a current through the coil, thus counteracting the torque due to the friction set up by the motion of the liquid. By acting on the other dials, he rapidly brings back the spot on the zero position and *reads on the milliammeter a figure which is proportional to the viscosity.* The whole operation takes less than 10 seconds.

By keeping the spot on the center of the scale (zero reading), all further changes in the viscosity of the liquid under observation are quantitatively read on the milliammeter. When a recording instrument is used, a curve expressing the evolution of the phenomenon can be obtained.

When it is desirable to secure more accurate figures the switch Sw (Fig. 1) is opened and an additional 100 ohms resistance is thereby introduced in the circuit. The terminals of this resistance are connected to a potentiometer. The potential drop in the resistance can thus be measured; it is proportional to the intensity of the current ($E = IR$). As R is constant, the drop is also proportional to the viscosity; and as the potentiometer gives three significant figures instead of one on the milliammeter (if the instrument allows readings to 0.1 mv) the sensitivity is increased one hundred fold.

The further advantage of this electromagnetic control is that the readings are made when the suspension is not subjected to any torsion (zero position), and that the instrument does not require a damping device as in the first model. The damping results automatically from the motion of the coil in the magnetic field. Moreover, a variable shunt makes it possible to pass instantaneously from one sensitivity to another, so that the range of the viscometer can be extended at will. The readings on the milliammeter are multiplied by the factor read on the shunt dial. These factors on the standard model are 0.02, 0.1,0.2,0.4,1.0,2.0. The first one, 0.02, is used for the viscosity of air, and 0.4 or 1.0 for that of serum.

Experimental

In order to save the reader's time, and to enable him to visualize a series of experiments at a glance, only charts will be given. The experiments chosen for publication are representative of the others, as no radical departure from the mean has been observed so far.

The experiments were carried on in the following way: the serum was poured into the cup (1 cc) and the bob lowered into it. The zero point being checked, the motor was started, and a first reading made at the starting temperature (between 20° and 25°). After checking the zero again, and repeating this measurement at least 3 times, with an interval of 5 minutes between readings, the heat was turned on. (A current of 1 ampere

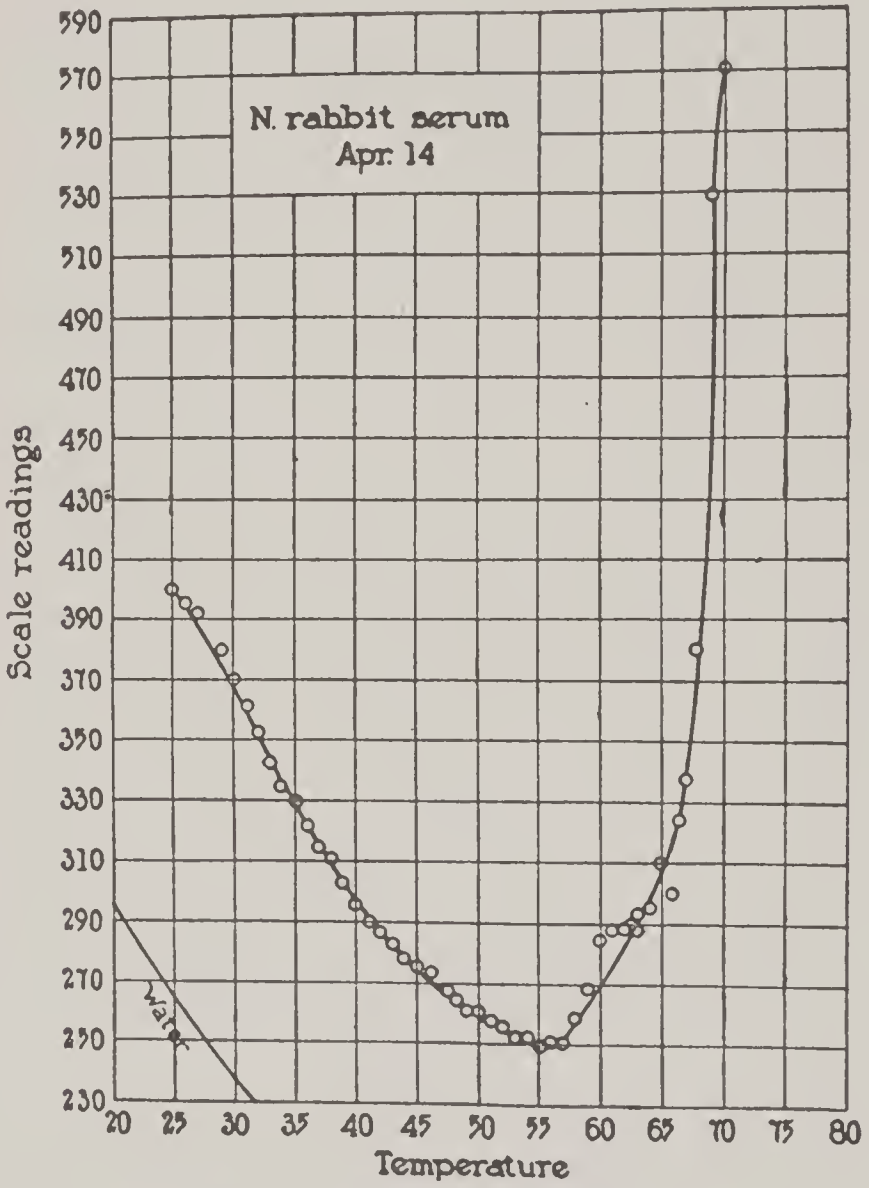

Fig. 2. Viscosity of rabbit serum as a function of temperature. An absolute minimum is observed between 55° and 56°C.

through a resistance immersed in the oil surrounding the cup brought the temperature up from 20° to 70° in about 45 minutes.) Then the readings were taken, and recorded simultaneously with the temperature. A telescope placed alongside the scale makes this possible. As a rule, unless some critical point was neared or some unforeseen phenomenon occurred,

readings were taken at every 2°. The current was left on until 70° was reached, in the first series of experiments (Figs. 2, 3, 4); in all other experiments reported in this paper the heat was stopped after a certain temperature had been reached (50°, 55°, 56°, to 60°) and the oil allowed to cool by itself. Usually, in order that cooling from, say, 55° to 20° should take about the same time as heating from 20° to 55°, cold water was made to

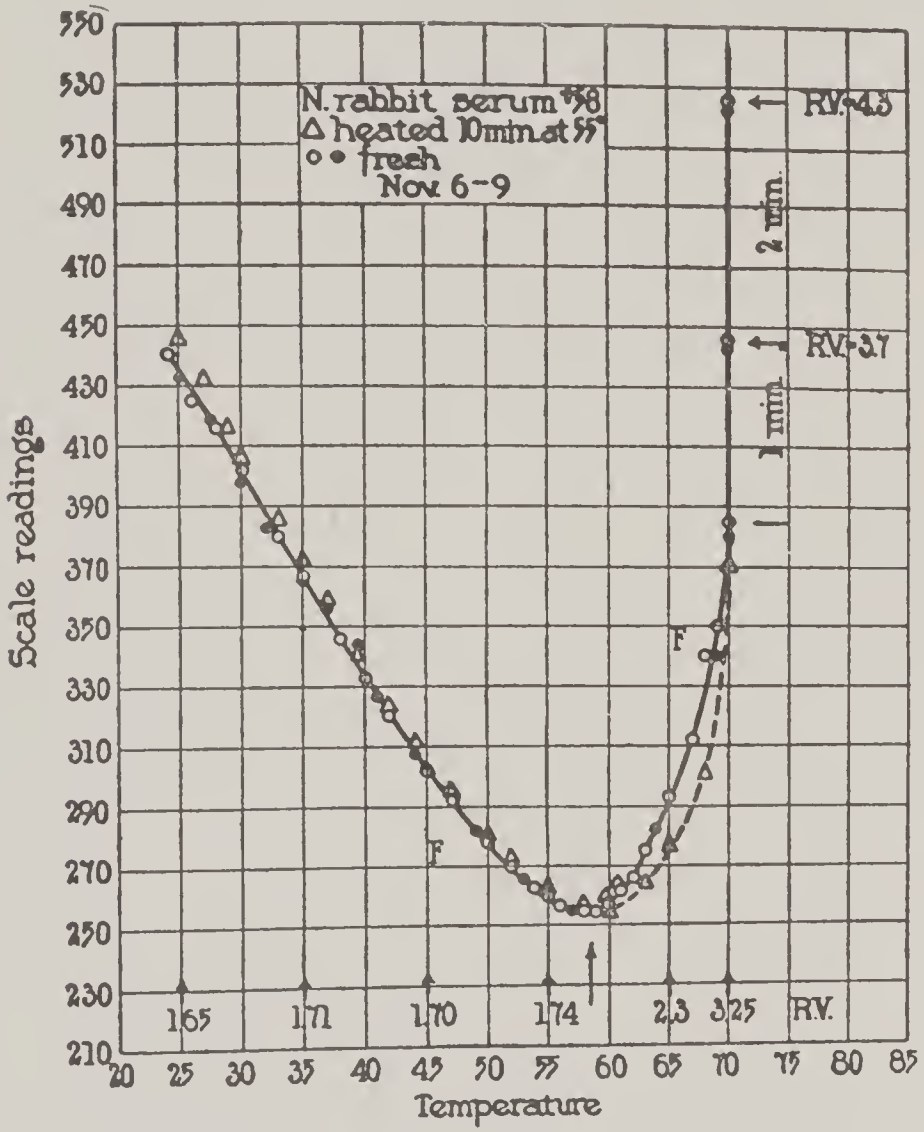

Fig. 3. Viscosity of rabbit serum as a function of temperature. The minimum is observed around 58°C. The letter F indicates the points where important and rapid fluctuations were observed. R.V. indicates the values of the relative viscosity with respect to water.

circulate in the double wall of the oil bath, and the rate controlled accordingly.

The first set of experiments is shown in Fig. 2.[3] The ordinates express the readings on the scale, and therefore are arbitrary figures, but propor-

[3] Lecomte du Noüy, *Jour. Gen. Physiol.*, 12, 363 (1929) and *Ann. Inst. Pasteur*, 42, 742 (1928).

tional to the absolute viscosities. In order to give an idea of the order of magnitude, the curve expressing the viscosity of water as a function of temperature is drawn in the figure.

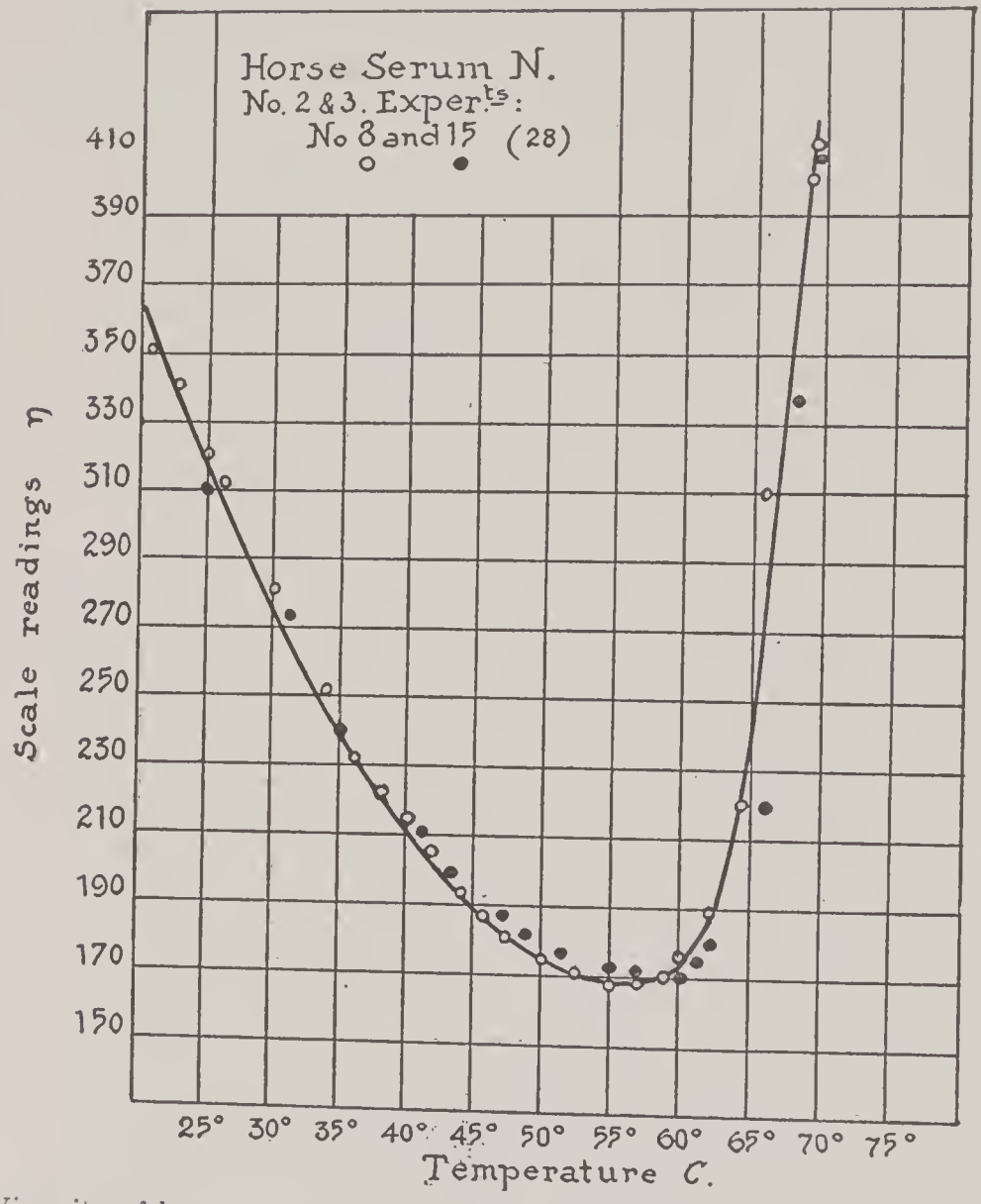

Fig. 4. Viscosity of horse serum as a function of temperature. The specific or relative viscosity $\frac{\eta}{\eta_0}$ being higher than that of rabbit serum (greater concentration of proteins) the sensitivity of the viscosimeter had to be decreased (by decreasing the rate of rotation of the cup). The scale readings do not correspond to the same values as on the other diagrams.

A glance at this chart immediately reveals the presence of a critical point, corresponding to an absolute minimum of viscosity, at a temperature near 56°. Had we not known beforehand that this was a critical temperature for serum, from a biological standpoint, we could not have failed to notice it from these curves.

Fig. 3 illustrates the same phenomenon. The turning point may vary from 56° to 58°. But in the great majority of cases, the viscosity reaches its minimum value between 56° and 57°, stays constant up to 58° sometimes up to 59°, and begins to increase more rapidly than it decreased before. From 62° to 65° the increase becomes very rapid, and it sometimes happens that the spot leaves the scale before 70° is reached. This is usually the case with horse serum (which is normally more viscous than rabbit

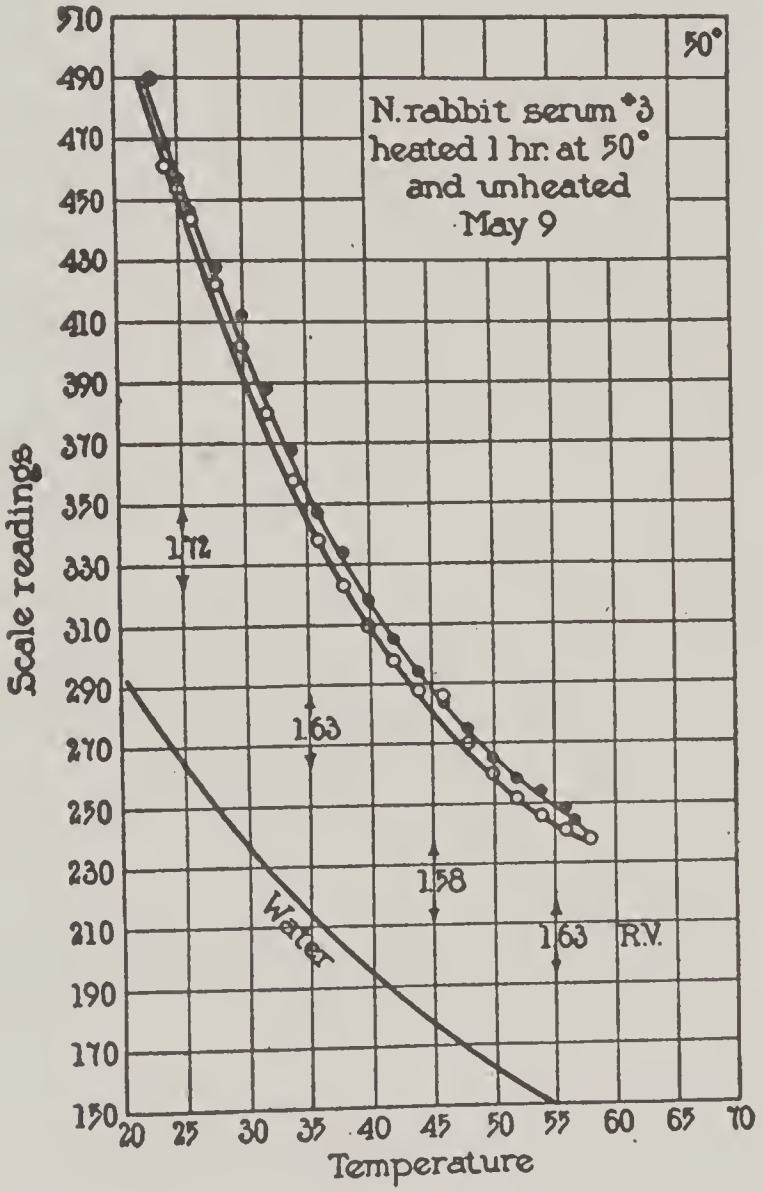

Fig. 5

serum). It happens rarely in the case of rabbit serum, the protein content of which is usually lower than that of serum from other animals, in particular horse serum. Fig. 3 shows that it was necessary to keep the temperature at 70° for 2 minutes in order to send the spot off the scale. It is to be noted that when fresh serum is used, *important fluctuations are fre-*

quently observed between 45° and *55°*. These were *never* observed when the serum had been subjected to a temperature of 56° even for 5 minutes, or when it was old. It appeared to us that it might be interesting to follow the phenomenon more closely, and to try to determine whether the temperature played a specific part in the changes of viscosity, or whether the time of heating was the chief factor—in other words, whether heating for

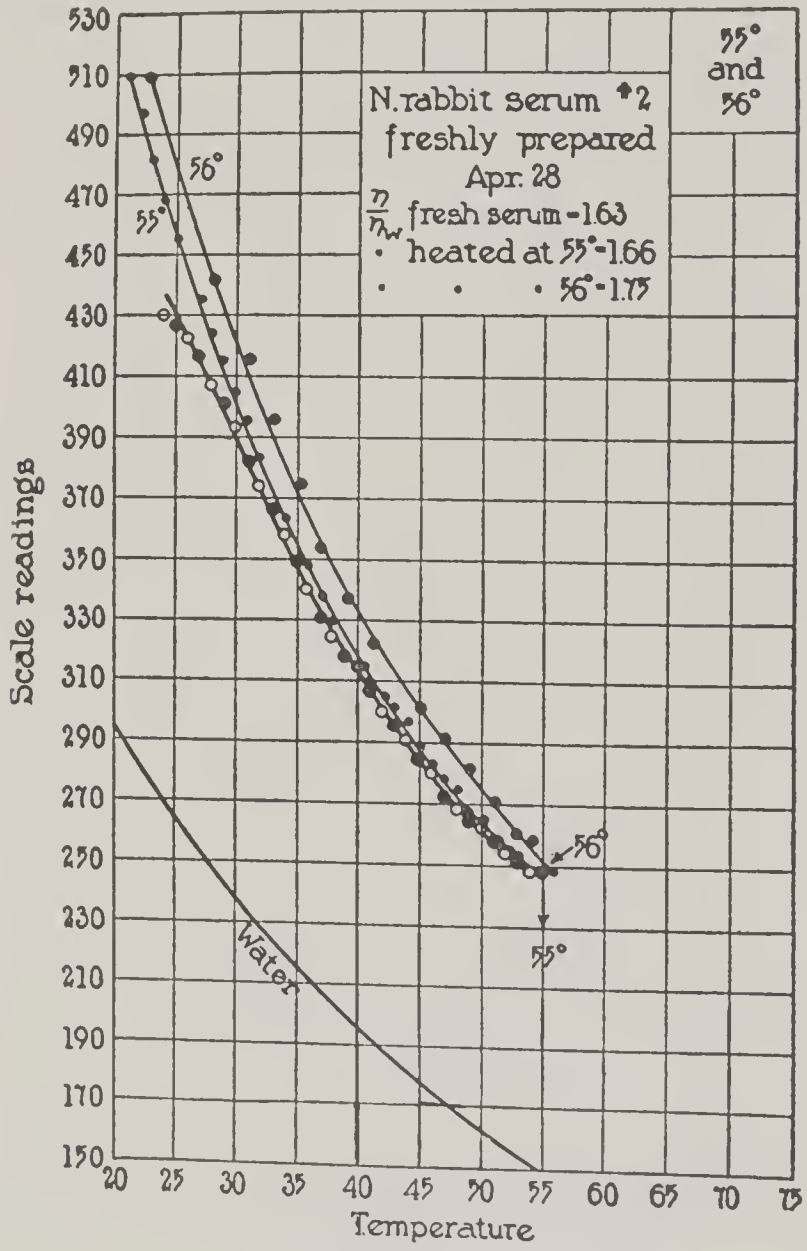

Fig. 6

15 minutes at 55° would produce the same increase in viscosity as 5 minutes at 60°, for example. The first experiment was made with a serum heated at 55° for 10 minutes in a sealed tube. It is obvious from Fig. 3 that there is no fundamental change in the curve; the two control curves (white and black circles) almost coincide with that of the heated serum.

A similar experiment is shown in Fig. 4 (horse serum). However, instead of carrying the heating to the time when the spot goes off the scale, it was stopped, and the liquid was allowed to cool, according to the technique previously described. The white circles express the values of the serum heated 1 hour at 50°. The black circles are the values taken by the heated serum, on cooling, after having been kept at 57° for 5 more minutes. In general, no difference can be detected between heated and unheated serum

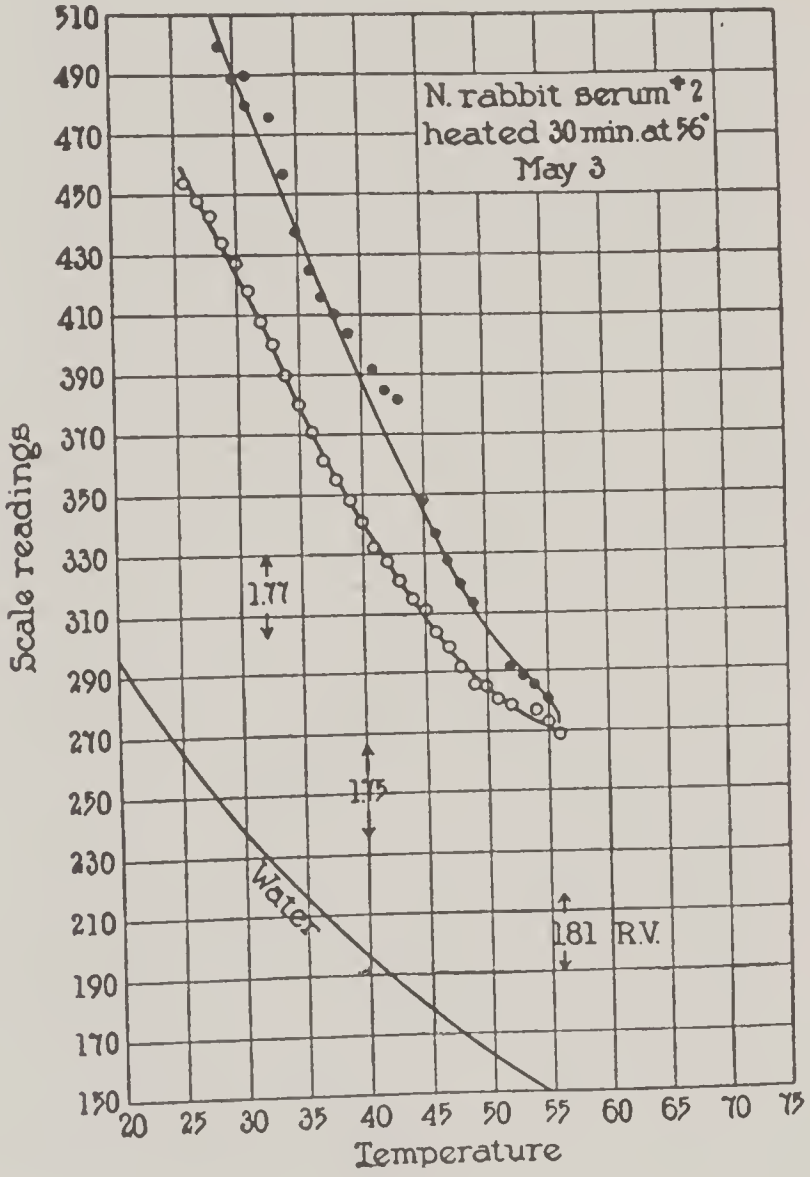

Fig. 7

when the serum is not heated above 55°. Up to 50° the curve expressing the viscosity of serum as a function of temperature is parallel to that of pure water, and its proteins play no part at all, or rather act only by their bulk, to displace the curve as a whole. From 50° on, a slight departure is observed; it goes on increasing until the minimum value is attained around 56°. Heating for 1 hour at 50° fails to alter permanently the viscosity of

serum. The phenomenon is entirely reversible, as in the preceding case (Fig. 5).

Heating for 15 minutes at 55° gives the same results. But as the heat was brought up to 56° in one series of measurements, while it was stopped at 55° in the other (Fig. 6), a small but marked difference could be detected between the two cooling curves. Taking evaporation into consideration, the slight increase in viscosity of the serum heated to 55° can be accounted for, but the difference between this sample and the one brought up to 56° is

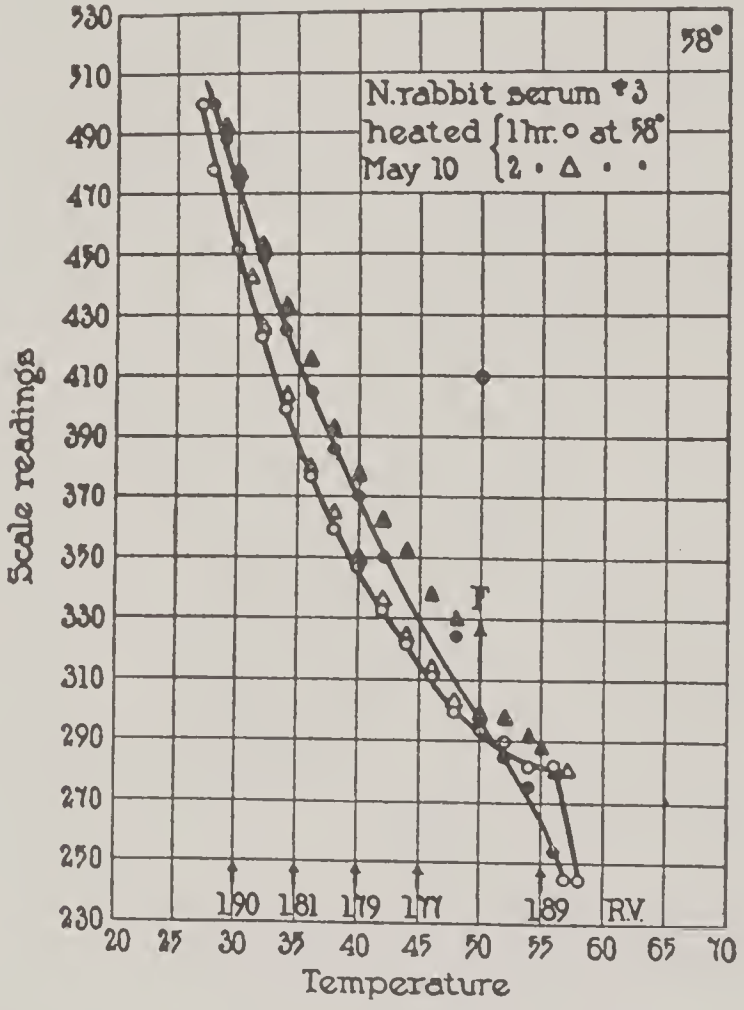

Fig. 8

due to something else. Here the phenomenon is no longer reversible; the relative viscosity reaches 1.70.[4] The same experiment was repeated with the same serum, heated for 15 minutes at 56° in a sealed tube. The mean viscosity was 1.70. The increase is small, but constant with this serum. It was not always observed with other sera. We can, therefore, state that

[4] The *relative* or *specific* viscosity is the ratio of the viscosity of the liquid experimented on (serum in the present case) to that of pure water at the same temperature, *i.e.* $\frac{\eta}{\eta_0}$. For the sake of simplicity, we often denote it as η.

from our experiments it appears that 56° is the lowest temperature at which an irreversible phenomenon affecting its viscosity occurs in rabbit serum in 15 minutes. However, an exception to this rule was once found. But if the heat is kept for 30 minutes at 56° (Fig. 7), the mean value of η climbs to 1.77, and higher still on cooling. On the other hand in certain cases, 5 minutes at 56° (dog serum) may bring forth no change at all.

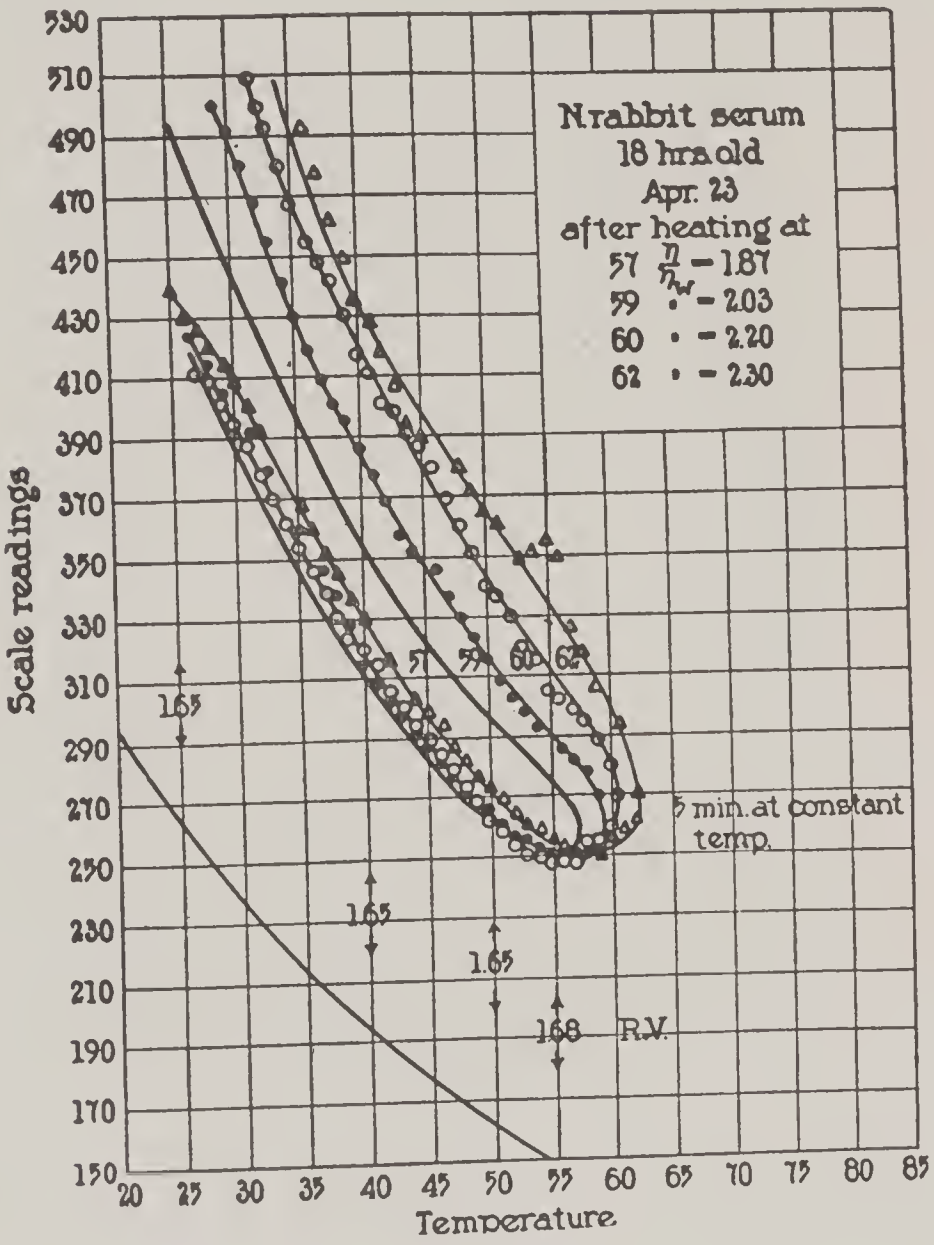

Fig. 9

Another serum, the viscosity of which was normally high, heated to 58° and cooled immediately after that temperature was reached, showed no modification. Serum 3 (η = 1.63) heated at 58° for 15 minutes, gave similar curves with η = 1.80. Half an hour at 58° brings the value of η up to 1.95.

Another serum heated for 1 hour and 2 hours, at 58° (Fig. 8) reaches a viscosity of 1.85 (mean value), with a maximum of 1.90. One hour's heating of this serum at 60° failed to affect it more than one hour at 58°.

Fig. 9 is self-explanatory. The serum was heated in the cup, for 5 minutes, in all cases. As the temperature goes up, the different samples

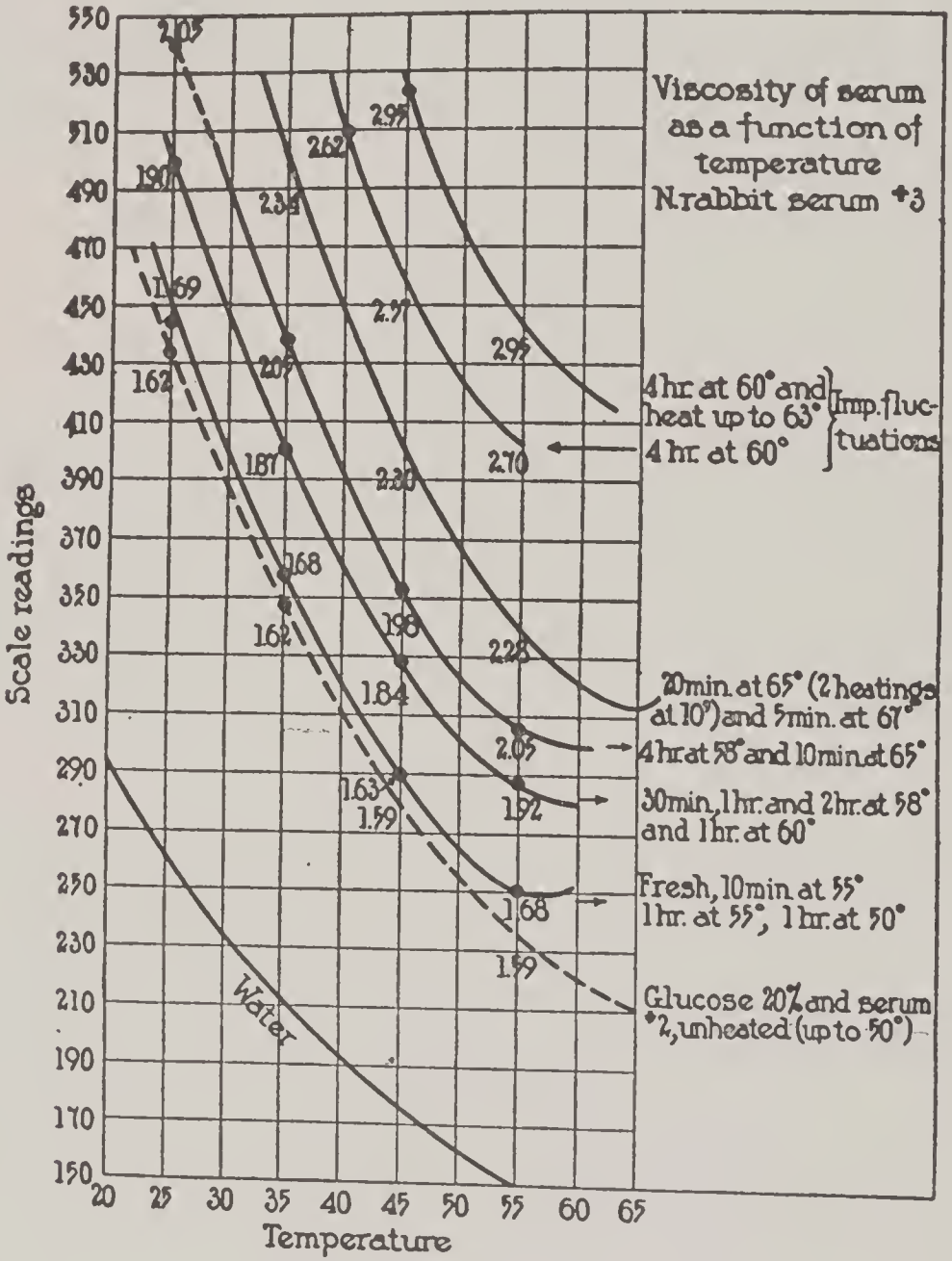

Fig. 10

show a similar behavior; but on cooling, the differences between the curves according to the temperature reached continue to increase. However, up to 62° no sign of structure in the liquid was found. The zero checked perfectly, and what was measured was true viscosity.

Fig. 10 summarizes for one animal a whole series of observations, show-

ing which combinations of temperature and of time determined the same increase in viscosity. The question then arises as to what the increase in viscosity is due to, and how it can be interpreted.

Discussion

In our experiments the concentration remains constant, yet the viscosity increases. On the other hand, Einstein[5] states that the degree of dispersion, *i.e.*, the size of the molecules or particles, is immaterial, and that viscosity can be expressed as a linear function of the volume fraction of the dispersed substance, according to the formula

$$\eta = 1 + 2.5\varphi \tag{1}$$

which is an approximation of the expression

$$\eta = \frac{1 + 0.5\varphi}{(1 - \varphi)^2} \tag{2}$$

where η stands for the relative viscosity of the suspension, and φ for the volume occupied by the dispersed substance expressed as a fraction of the total volume of the solution. These equations, however, fail to express the experimental facts. The reason for this lack of agreement is not clear. But Kunitz has proposed an empirical formula which applies remarkably well to a number of widely different cases, including lyophilic and lyophobic sols, and within large limits of concentration. This formula is[6]

$$\eta = \frac{1 + 0.5\varphi}{(1 - \varphi)^4} \tag{3}$$

The problem we are facing can be stated as follows: Assuming that Einstein's view concerning the part played by φ as defined above is correct and that the degree of dispersion is immaterial, we can use formula (1), and compute the values of φ, and consequently determine φ/C, which expresses the specific volume of the solute (proteins). These values divided by the specific volume of the dry proteins should give the amount of hydration of the proteins in solutions, at temperatures up to 55°, and its increase as a function of temperature. But we know that this formula does not apply to lyophilic sols at high concentration; consequently, we cannot rely on the figures at all.[7]

[5] Einstein, A., *Ann. Physik.*, 19, 289 (1906); 34, 591 (1911).

[6] Kunitz, M., *J. Gen. Physiol.*, 9, 715 (1926).

[7] The influence of the charge of colloidal particles on the viscosity of the sol has been taken in consideration, especially by von Smoluchowski [*Kolloid Z.*, 18, 194 (1916)], who enlarged Einstein's formula, which became:

$$\eta_s = \eta_m\left\{1 + 2.5\varphi\left[1 + \frac{1}{\lambda\eta_m r^2}\left(\frac{D\zeta}{2\pi}\right)\right]\right\}$$

On the other hand, we can apply Kunitz's formula (Table 1) which we know fits the experimental facts very satisfactorily; but then the main assumption of Einstein concerning the role of the degree of dispersion, which is a consequence of his mathematical derivations, may not hold any longer. Therefore, it does not seem possible, at present, to decide whether the figures computed in this way express quantitatively the increase due to hydration alone, or whether some other phenomenon is also responsible for it to a certain extent. However, column 6 has been tentatively designed as "hydration."

Table 1. Viscosity of Rabbit Serum 3, Heated at Different Temperatures, and Values of φ and φ/C Computed from Kunitz's Formula (see Figs. 9 and 10).

C = Mean Concentration of Proteins in the Serum = 6.5 per cent

1	2	3	4	5	6	7
Relative Viscosity $\frac{\eta}{\eta_0}$	φ (%)	Specific Volume φ/C	Specific Volume of dry Proteins	Ratio: $\frac{\text{Col. 3}}{\text{Col. 4}}$	Increase % of specific volume (Hydration?) (%)	Increase in Hydration due to Heating (% unheated)
1.65	10.7	1.645	0.785	2.09	109	unheated
1.69	11.2	1.720	"	2.19	119	serum
1.88	13.3	2.030	"	2.58	158	39
2.03	14.8	2.275	"	2.90	190	71
2.30	17.2	2.650	"	3.37	237	118
2.62	19.8	3.050	"	3.88	288	169
2.95	21.8	3.350	"	4.27	327	208

All that can be said is that it expresses the increase per cent of the specific volume of the serum proteins. It is interesting to note that the figures expressing "hydration" obtained from Einstein's formula (3) are larger (3.71 times) than those obtained from Kunitz's formula (1), and that this ratio remains constant up to a viscosity of 2.03. From this figure up the discrepancy begins and increases rapidly with increasing viscosity.

Fig. 10 suggests another observation: the dotted curve (viscosity 1.62) was obtained with a sugar solution (glucose 20 per cent). The concentration is 3 times that of serum. Hence, serum may be said to behave exactly like a true solution, as a function of temperature, up to nearly 55°. It is surprising that such a concentrated solution of proteins should have such a low viscosity. When, by diluting the serum, its specific volume is made

However, our results are not in accord with this formula, which should lead to a lower viscosity as the size of the particles increases. We observe the contrary, and there seems to be no doubt that, after heating, the size of the particles increases, since coagulation is the limit of the phenomenon. Perusal of Freundlich's excellent discussions of the subject in general (*Colloid and capillary chemistry*, pp. 367 and following, 539, etc.) will repay the reader.

equal to that of the sugar solution, its viscosity is much less than that of the latter.

The study of the viscosity of blood serum as a function of temperature, performed by means of a new instrument, has revealed the following facts:

(1) The curve representing the variations in viscosity shows an absolute minimum located around 56°.

(2) On either side of this minimum viscosity increases, but much more rapidly with increasing temperatures.

(3) When a serum is heated to 55° in a sealed tube, its viscosity is not manifestly increased even though the heating is kept up for one hour.

(4) In general, beyond 56° an irreversible increase of viscosity takes place even when this temperature is maintained for only 5 minutes. However, a few exceptions have been observed in which this phenomenon begins only around 58°.

(5) Each degree above 56° brings forth an irreversible increase in the viscosity of serum; this viscosity can thus be almost trebled without trace of coagulation and there is no indication of a structure in the serum.

(6) By applying to this phenomenon the ideas of Einstein and by using the formula of Kunitz we have computed the increase in the specific volume of the proteins in a given serum. If we admit, as seems logical, that this increase is due to the fixation of water on the serum molecules, these figures express the degree of hydration at different temperatures. At normal temperature, and up to 55°, the hydration would be around 120 per cent. It reaches more than 300 per cent under certain conditions, and probably goes on increasing. But the viscosimetric method which we used at the time made it difficult to measure greater viscosities. The new instrument described in this chapter (Fig. 1) would make these measurements easy. It will be seen in the succeeding chapters that we circumvented this problem by using an optical method.

Chapter 4

Rotatory Power and Rotatory Dispersion of Serum As a Function of Time and Temperature

The preceding facts led us to think that another, more fundamental problem existed which was the *immediate* consequence of heating at a given temperature. In that case, this phenomenon would be the determining cause both of the changes in the biological properties and of the modifications in the nature of the relations between the affected molecules and the molecules of the solvent. These transformations should also bring about a difference in the interrelations between the protein molecules. It was logical to assume that this fundamental phenomenon, common cause of the biological and physico-chemical alterations, was of a chemical nature, *i.e.*, that it affected the very structure of the serum molecules.

If, besides the minimum of viscosity and the destruction of the complement, a third phenomenon, revealing a profound alteration of the structure of the molecule, could be shown to exist in the neighborhood of the critical temperature, we could deduce therefrom that the two first phenomena are direct consequences of the third. It was with this idea in view that we studied the rotatory power of different sera, at temperatures ranging from 0° to 70°.

Technique and Experiments[1]

The measurements were made with the Jobin & Yvon high-precision, half-shade polarimeter, which gives readings accurate to 1/100th of a degree. All the cells employed were 10 centimeters long. The source of light was a mercury-vapor lamp. The light rays passed through a monochromator. The control of the monochromator was close at hand so that the wave length could be rapidly changed in order to study the *rotatory dispersion.* The indigo line (λ = 4,358 Angstroms), though very intense, was completely absorbed. We were, therefore, obliged to use the green line (λ = 5.461 Angstroms), the yellow lines (λ = 5,769 and 5,790 A together), and sometimes the red line (λ = 6,234 A). The serum was contained in carefully stoppered tubes, or more generally in sealed tubes and kept at the desired temperature in large Thermos bottles equipped with agitators. The temperature, which

[1] Lecomte du Noüy, *Ann. Inst. Pasteur*, 43, 749 (1929).

was frequently controlled, did not vary by more than $\pm 0.25°$, during experiments which lasted about one hour.

For the second series of experiments (coefficient of temperature) mentioned in this chapter, it was necessary to set up a special apparatus in the following manner. We used a 10-centimeter water-jacketed cell of small diameter equipped with a thermometer. The water was electrically heated and kept moving in a closed circuit by a small pump. The pump kept the liquid in rapid circulation and the temperature was regulated by an appropriate rheostat. When starting with low temperatures, the water was circulated through a coiled metallic tube immersed in melting ice or in a refrigerating mixture. The passage from 0° to 55°, was continuous and as slow as desired. When the temperature of the room was reached (about 20°) the electric heating was brought into play. By cutting off the current it was possible to keep the temperature sufficiently constant for several minutes owing to the calorific inertia of the whole and the isolating precautions which were taken.

The results of the experiments will be presented in the following order: (1) influence of time; (2) influence of temperature, from 0° to 55° (coefficient of temperature); (3) influence of a given temperature as a function of time; (4) rotatory dispersion.

(1) Influence of Time. To begin with, it was necessary to study the spontaneous changes which might affect the rotatory power of serum as a function of time. (As is well known, serum is always levo-rotatory.)

With this end in view the rotatory power was measured at different intervals of time. The figures in the tables and diagrams correspond to the value of the change in the angle of the polarization plane. As we were interested only in the variations, we did not think it worth while to calculate, for each measurement, the specific rotatory power expressed by

$$[\alpha]^t_\lambda = \frac{\alpha}{L\gamma}$$

where α = the change in the angle of the polarization plane; L = the length of the cell *in decimeters*, and γ = the concentration in grams per cc of solution.

The specific rotatory power varies according to the sera. Its mean value is $[\alpha]^{20} = 58$ for the green line ($\lambda = 5{,}461$ A), $[\alpha]^{20} = 49$ for the yellow line (λ mean $= 5{,}780$ A), and $[\alpha]^{20} = 40$ for the red line ($\lambda = 6{,}234$ A). The extreme values observed for the green line were $[\alpha]^{20}_{5461} = 52$ and $[\alpha]^{20}_{5461} = 62$ (normal horse serum).

The sealed tubes were divided into two lots. One was kept in an ice-box at $+2°$, and the other simply in a drawer at normal temperature.

This experiment shows that, under these normal conditions, no changes

Table 2. Serum No. 889, Horse, Exp. No. 40, λ = 5,780 A (yellow), Mean.

Time	0	1h.	2h.30	3h.30	4h.30	5h.30	6h.30	7h.30
α	3.68°	3.68°	3.66°	3.66°	3.66°	3.66°	3.66°	3.66°

superior to acceptable experimental errors appear in 7½ hours. However, occasionally these modifications can attain a more important value, as is proved by Table 3 which covers only 35 minutes.

Table 3. Normal Horse Serum No. 2. Exp. No. 42.
Values of α read successively between 3h.15 and 3h.50, λ = 5,780 A

Sample in ice-box α	Sample at room temperature α
3.80°	3.85°
3.90°	3.85°
3.88°	3.90°
3.87°	3.90°
3.87°	3.88°

The differences here attain 0.08°, for the sample in the ice-box.

Table 4 gives the results of an experiment extended over fifteen days.

It can be seen that the figures show a maximum deviation of 0.11°. These deviations are not entirely due to errors in the readings, for these

Table 4. Normal Horse Serum No. 1. Exp. No. 42.

Days	1	3	6	9	13	15	Maximum deviation
Room temp:							
λ = 5,780 A	3.83°	3.88°	3.90°	3.90°	3.85°	3.86°	0.07°
λ = 5,461 A	4.45°	4.43°	4.51°	4.45°	4.41°	4.51°	0.10°
Ice-box:							
λ = 5,780 A	3.86°	3.85°	3.88°	3.79°	3.89°	3.86°	0.10°
λ = 5,461 A	4.51°	4.40°	4.46°	4.40°	4.43°	4.42°	0.11°

errors are never greater than ± 0.03°. They are, therefore, determined by spontaneous changes in the rotatory power of the serum which, in certain cases only, oscillates around a mean value. But generally—up to 40 days—time does not seem to modify systematically the rotatory power of a given serum. A decided darkening of the solution in polarized light can be observed after a lapse of time which varies with the sample of serum (generally after 16 days). Occasionally, a slight but permanent increase in the value of α can be observed at the end of fifteen or twenty days.

This point being established, we know the order of magnitude of the experimental errors which escape our control, and we can safely start the study of the action of temperature.

(2) Temperature Coefficient. Table 5 gives the results of a series of experiments made on normal serum. The figures represent the values of α obtained by subtracting the dial reading from 360°.

The serum was left in the cell on the polarimeter, and the next day (22 hours later) the experiment was repeated, as the serum had spontaneously cleared (Table 6).

It is evident that, at the three wave lengths employed, there is no systematic change in the rotatory power up to 54°. The rotatory dispersion is,

Table 5.

Temp. (° C)	22°	25°	31°	35°	39°	45°	Observations
Green line	4.20°	4.17°	4.20°	4.25°	4.23°	4.22°	Readings difficult beyond 35° because of darkening of the field
Yellow line	3.60°	3.62°	3.60°	3.60°	3.62°	3.64°	
Red line	2.93°	unreadable					

therefore, also constant and the partial dispersion between the yellow and green lines, *i.e.*, $\alpha_{5461} - \alpha_{5780}$, has a mean value of 0.57°. This same serum, which had been kept in its tube (and which therefore had been subjected to successive heating at all the temperatures indicated in Table 6) was examined again 24 hours later at room temperature. The values obtained were: $\alpha = 4.55°$ for the green, and $\alpha = 3.90°$ for the yellow. It can be seen that this time the heating had determined an irreversible phenomenon in the serum, the angle increasing from $\alpha = 4.21°$ (mean value) to 4.55° and from $\alpha = 3.63°$ (mean value) to 3.90°. The rotatory dispersion is also affected and its partial value $\alpha_{5461} - \alpha_{5780}$ is 0.65° instead of 0.57°.

Table 6.

Temp. (° C)	22°	25°	30°	32°	36°	43°	49°	52°	54°	60°	5 m. at 60°	10 m. at 60°
Green line	4.23°	4.21°	4.23°	4.23°	4.16°	4.22°	4.22°	4.22°	4.22°	4.28°	4.33°	4.43°
Yellow line	3.67°	3.65°	3.67°	3.65°	3.63°	3.64°	3.66°	3.67°	3.68°	3.71°	3.78°	3.85°
Red line	2.94°		3.07°			3.05°		3.03°				

As we thought that the slight variations observed between 22° and 54° might be due to errors in the readings, this experiment was repeated under better conditions by neglecting, momentarily, the rotatory dispersion, that is, by working with a constant wave-length. When the angle of rotation, corresponding to a perfect matching of the fields, is set on the instrument, the worker can immediately distinguish small differences when they occur, but is not constantly obliged to equalize the fields in different colors, a procedure which gives rise to errors, varying with the personal coefficient.

All these measurements were made with the yellow lines, as this wave length is most frequently used, and started at the temperature of 10° (Table 7).

Similar results were obtained, under the same conditions, when using green light.

Table 7. Duration of Experiment 1 Hour

Temp. (° C)	10°	20°	22°	35°	45°	50°
Yellow 5,780 mean Observations	α 4.03°	4.03° obscure	4.03° clear	4.03°	4.03°	4.03°

It can therefore be stated that the rotatory power of serum is constant for $\lambda = 5.461$ (green) and $\lambda = 5.780$ (mean yellow), and that this is also true for the rotatory dispersion, from 10° to 50°. *There is no temperature coefficient*, which shows that there are no changes of a chemical nature in the substances in solution for a heating time of up to thirty minutes. It is extremely probable that this applies to all the visible spectrum, as the few measurements which could be performed with the weak red line were in accord with the others.

The phenomenon of the darkening of the fields which occasionally happens without apparent cause, or under the influence of heating, and on which we shall dwell later, seems to be of a purely physical nature and does not affect the readings in any way. A curious fact is that this darkening occasionally decreases spontaneously. It may be linked to an unknown structural or dispersive condition of the solution.

(3) Influence of Temperature as a Function of Time. The fact having been established that serum was not affected optically by progressive

Table 8. Prolonged Heating at 50°. (Exp. No. 20). Normal Horse Serum No. 1.

Time	0 (unheated)	10 min.	20 min.	40 min.	60 min.	2 hours
Green	4.24°	4.24°	4.29°	4.31°	4.37°	4.35°
Yellow	3.68°	3.68°	3.72°	3.72°	3.77°	3.77°
Red	2.95°	3.03°	3.10°	3.13°	3.13°	3.13°

heating up to 50°, it became necessary to study the influence of prolonged time of heating. For the sake of clearness and in order to avoid tables of figures in which the results of an experiment as a whole do not stand out, we shall only give a small number of detailed experiments and replace the others by curves which enable the reader to take in a series of measurements at a glance (Table 8).

This experiment shows that up to 20 minutes of heating the value α is about constant (the difference being of the order of magnitude of experimental error). After one hour there is a slight increase, the mean value of which is 0.10°. Two hours of heating do not modify these values. This increase is not always observed, and in none of our experiments was the increase greater than that which could be attributed to experimental error.

The results of heating at 52°, 54°, 56°, and 58°, are given in Fig. 11. In order to be able to compare the curves we drew them all on the same

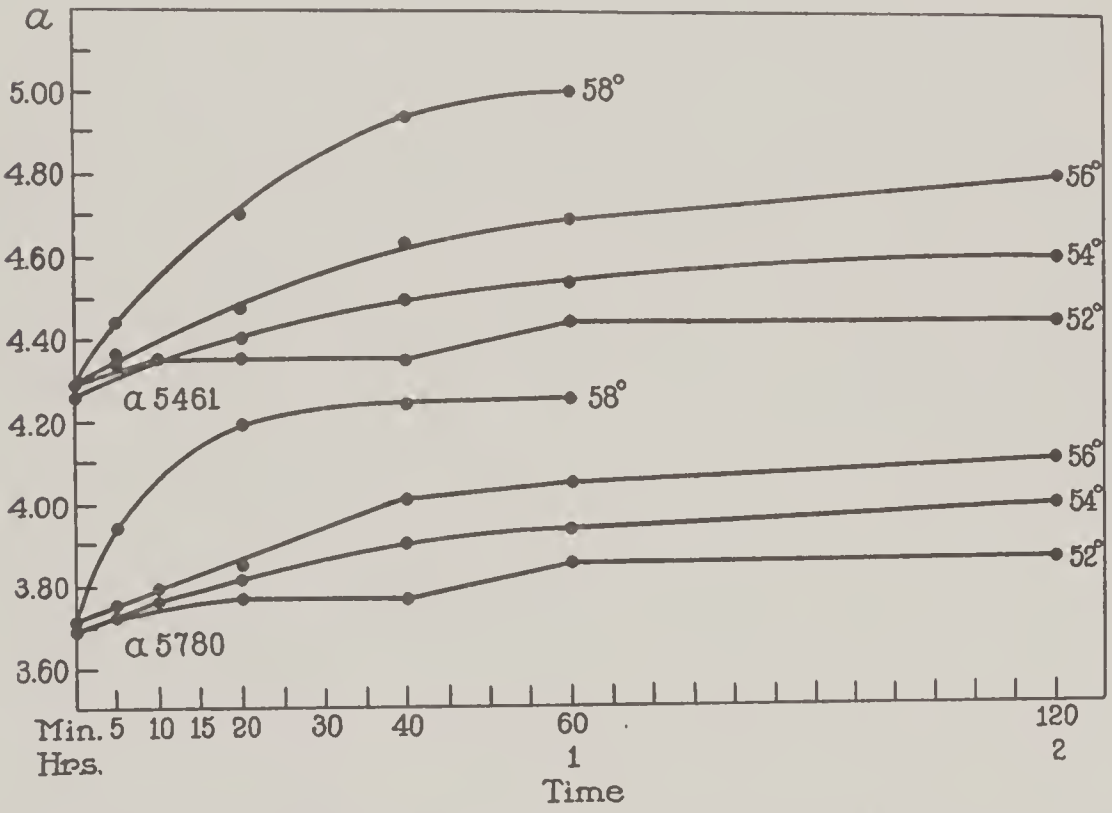

Fig. 11. Normal horse serum (Exp.N°22.) Variations of α as a function of time, at different temperatures (from 52° to 58° C.). λ=5461 A. and 5780 A.

chart and, as the value of the rotatory power of unheated serum is not always the same, we displaced the whole curve, when necessary, by an amount equal to the difference, so as to give them all about the same starting point. For instance, the curve for 58°, which showed the greatest deviation, was raised by 0.08°.

These curves show that above 58° (in this experiment) the rate of the phenomenon increases rapidly.

Fig. 12 shows the aspect of the curves between 60° and 66°. Above 58° it is impossible to make measurements on serum heated for 60 minutes, and the darkening of the fields increases more rapidly in proportion to the rise in temperature. This phenomenon limits the observation, and every-

thing takes place as if the angle α could not increase beyond a certain value for a tube of a given length. As we have already pointed out, this darkening is a consequence of heating, but does not affect the readings. When a clear serum becomes dark during an experiment, which sometimes happens, α keeps the same value until the moment when the reading becomes impossible.

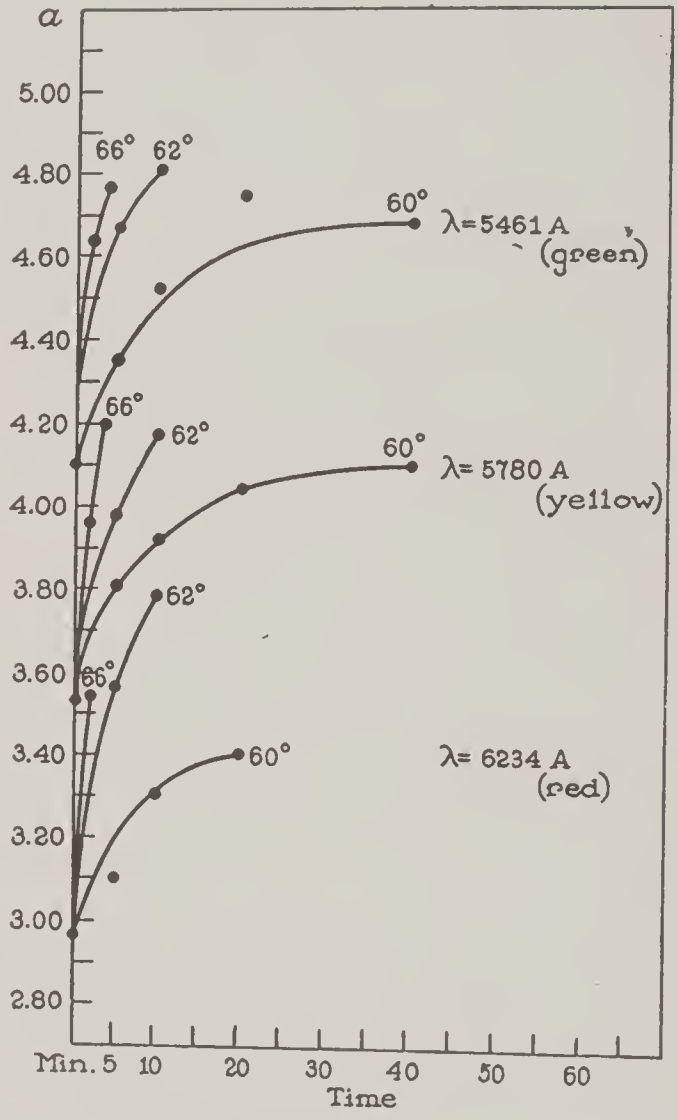

Fig. 12. Normal horse serum (Exp. N°22 cont.) Variations or α as a function of time, at different temperatures (from 60° to 66° C.). λ=5461, 5780 and 6434 A.

Certain sera are normally opaque, and it sometimes happens that they clear spontaneously. Fig. 13 indicates the duration of the time of heating before absolute darkening sets in for a normal horse serum. The regular, geometric aspect of the curve shows that this is a well defined phenomenon, correlative to heating; but actually it is impossible to know more about it. This problem deserves further investigation.

In order to show more clearly the general aspect of the phenomenon of the increase in rotatory power, we have plotted the increases of α as a function of temperature during the first minute of heating (Fig. 14). This can

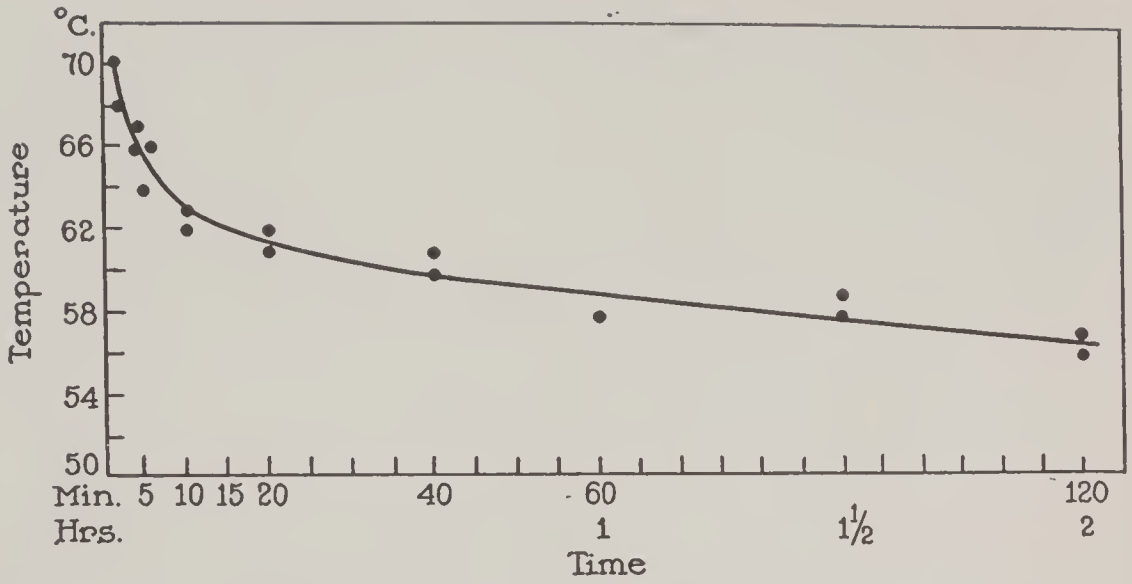

Fig. 13. Time of heating after which readings are still possible before complete darkening of the fields in the polarimeter.

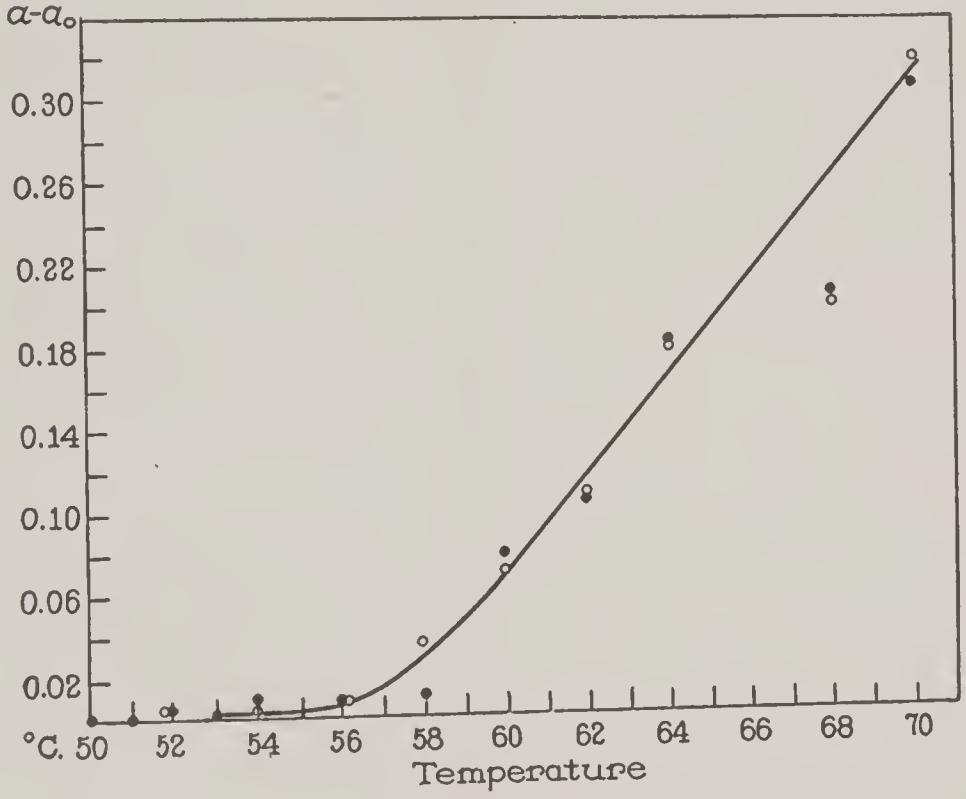

Fig. 14. Increase of α per minute ("rate" of modification of the rotatory power) during first 5 minutes of heating.

be taken as expressing the rate of increase per minute during the first 5 minutes if we admit conventionally that the increase is proportional to the time during this period. The figures are obtained by dividing the increase during five minutes by 5. We thus introduce a slight error which is negligible in the present case. One can see that the increase of α reaches a value

greater than that of the experimental errors around 57°, and that the acceleration becomes very nearly constant around 58°. These facts will be discussed later.

The increases in α for a normal serum heated for 5, 10, 20, 40, 60, and 120 minutes are shown in Fig. 15. It can be seen at a glance that the alteration in the serum after heating depends less on the time of heating than on the temperature. Serum must be heated for *two hours at 55°* in order to obtain

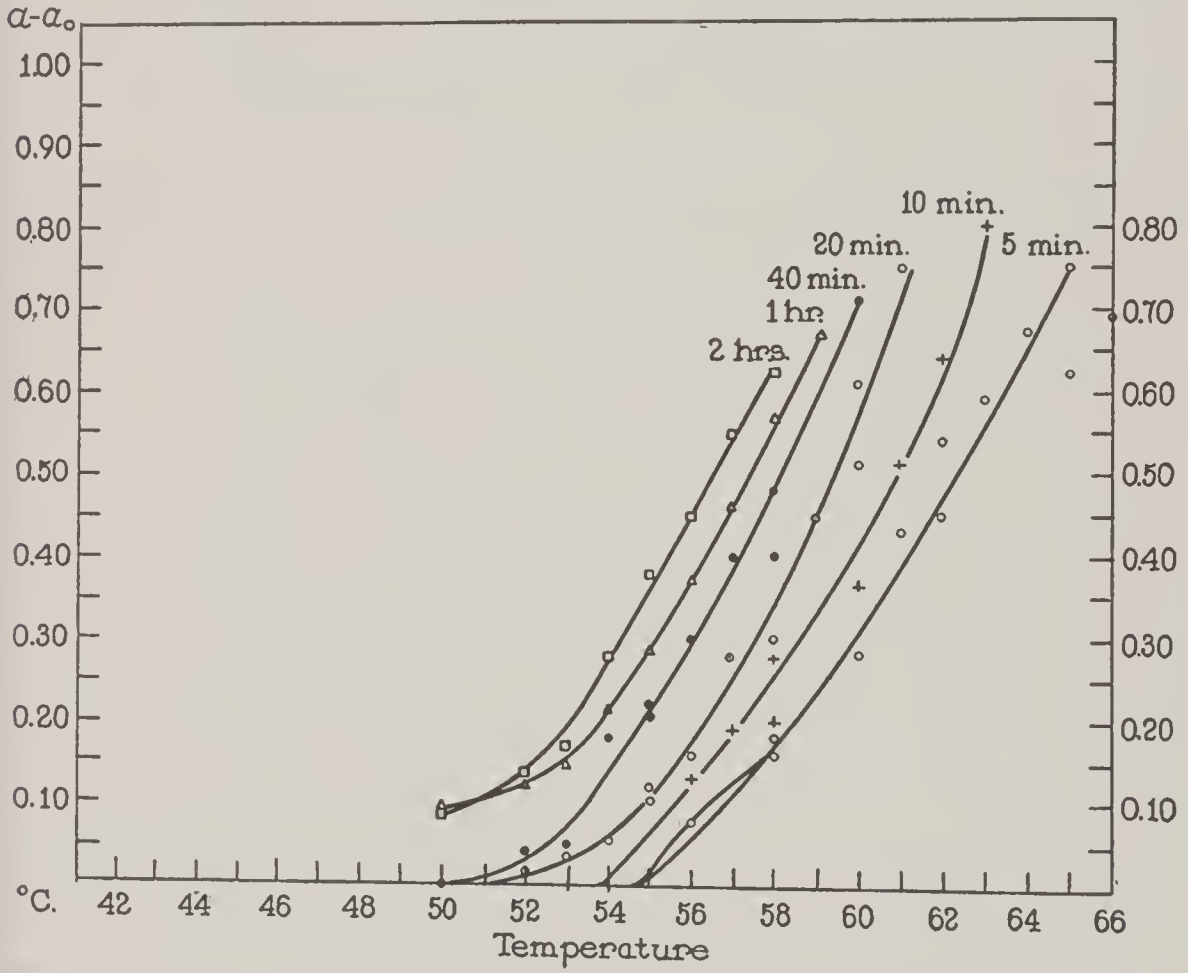

Fig. 15. Increase of α for different times of heating from 5 minutes to 2 hours as a function of temperature.

approximately the same results as those brought about by heating *five minutes at 61°*. Two hours at 58° are equivalent to five minutes at 63°, as far as α is concerned.

All the preceding results were obtained with normal serum. It seems that immunized serum reacts in a similar way, as can be gathered from Figs. 17 and 18 when compared to Fig. 16. They also indicate that the increase in rotatory power varies considerably from one animal to another, but that the rate of increase is approximately the same. Indeed, in Figs. 15, 16, and 17, the three corresponding curves can be superposed (heating

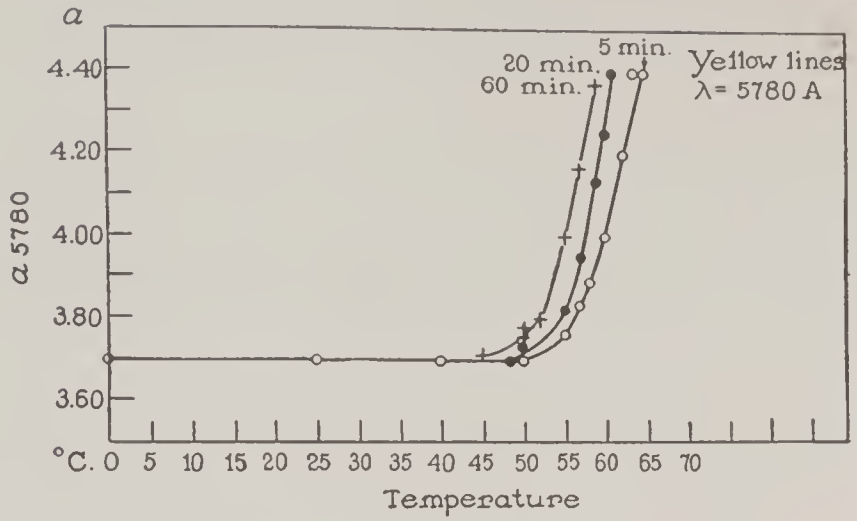

Fig. 16. Increase of the rotatory power of horse serum (α) after 5, 20 and 60 minutes heating as a function of temperature.

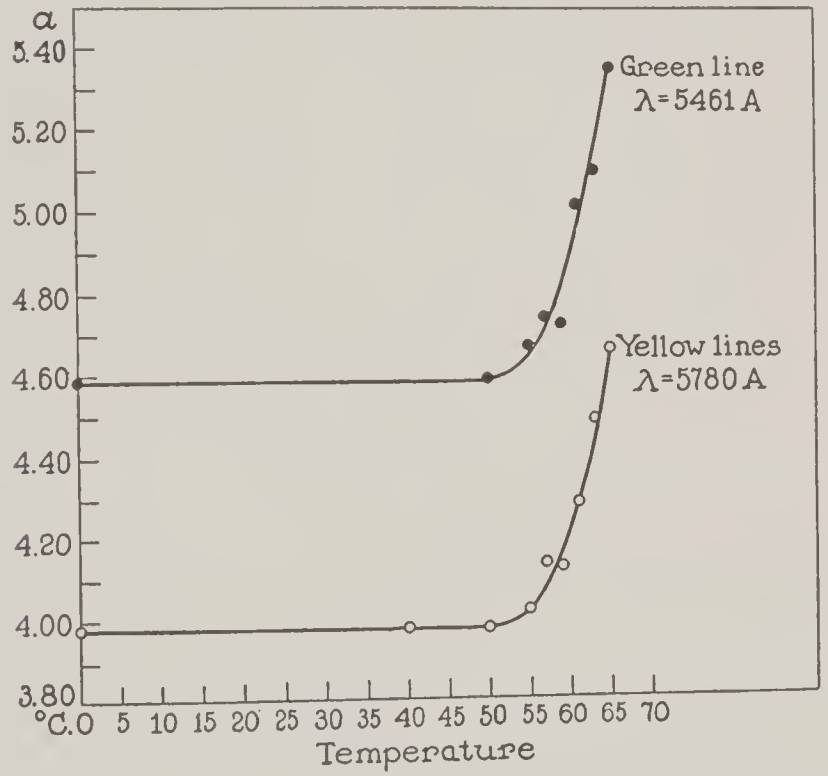

Fig. 17. Increase of the rotatory power of immunized horse serum (Perfringens) as a function of temperature. 5 minutes heating.

for 5 minutes) if one takes into account the displacement due to the different concentrations in proteins. These curves clearly indicate the existence of a critical temperature in the serum.[2]

[2] A serum kept for 48 hours in an incubator at 38° does not show any increase of α greater than that which may result from experimental error (horse). After 96 hours, although the serum is absolutely clear to the eye, it is generally completely obscure in the polarimeter and a measurement becomes impossible.

(4) Rotatory Dispersion. The curves in Figs. 17 and 18 were obtained with monochromatic lights of different wave lengths and indicate the general aspect of the phenomenon. If, as was done above, we agree to call partial rotatory dispersion the difference between the values of α in green

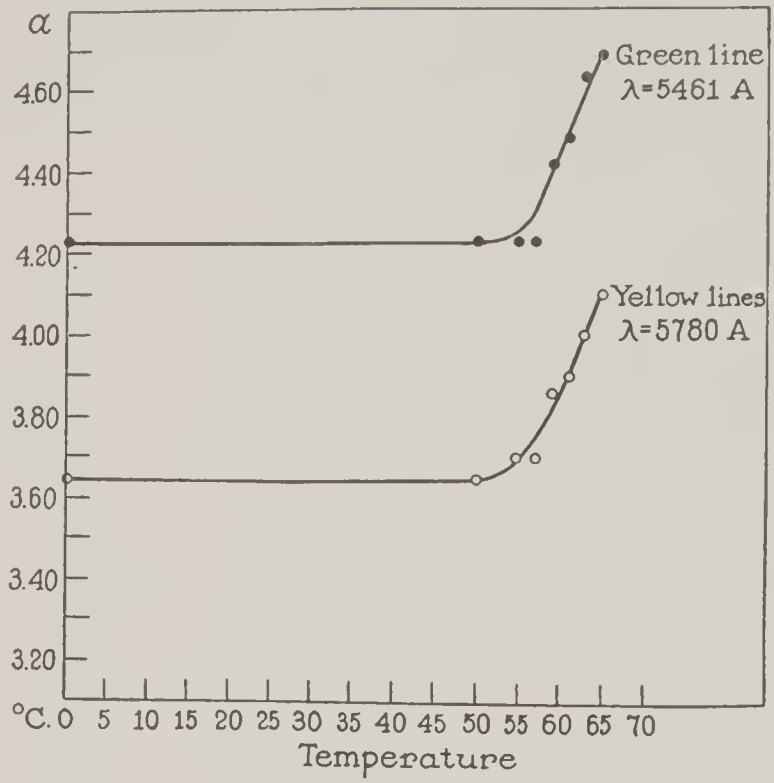

Fig. 18. Increase of α as a function of temperature. Immunized horse serum (cobra venom). 5 minutes heating.

monochromatic light (λ =5,461 A) and in yellow monochromatic light (λ (mean) = 5,780 A), we obtain, for the mean value of twenty-two series of experiments, the figure:

$$\alpha_{5461} - \alpha_{5780} = 0.543°. \quad \text{(Table 9.)}$$

Table 9. Observed Values of the Partial Rotatory Dispersion of Normal Horse Serum

0.48°	0.41°
0.44	0.55
0.55	0.54
0.53	0.57
0.57	0.57
0.60	0.56
0.49	0.60
0.53	0.56
0.53	0.52
0.65	0.55
0.55	0.54

Mean Value = 0.543° (unheated.)

Table 10 gives the values of $\alpha_{5461} - \alpha_{5780}$ obtained with heated sera. As the amplitude of the phenomenon is not very great, we have recorded in this table the highest values, in each experiment, which preceded the darkening of the fields. These values correspond to different times of heating and temperatures. It is nevertheless clear that the increase in the partial rotatory dispersion varies relatively little. Occasionally a value of 0.90° for a heating of 3 minutes at 67° was observed, and a low value of 0.48° for a heating of 2 minutes at 70°. It is actually impossible to understand clearly the mechanism of these phenomena, and we must content ourselves with very approximate statistical observations.

Table 10

Serum No.	(° C) Temp.	Time of Heating (hours)	$\alpha_{5461} - \alpha_{5780}$
1	54	2.00	0.61
2	56	2.00	0.64
3	56	2.00	0.64
4	57	2.00	0.60
5	58	1.30	0.66
6	58	1.00	0.65
7	59	1.30	0.76
8	59	1.00	0.74
9	60	0.20	0.70
10	61	0.40	0.71
11	61	0.20	0.71
12	61	0.20	0.75
13	61	0.20	0.71
14	61	0.05	0.66
15	62	0.05	0.66
16	63	0.05	0.74
17	64	0.05	0.75
18	64	0.05	0.73
19	65	0.05	0.71
20	66	0.05	0.66
			13.79/20 = 0.689°

It is clear that there has been an increase of the partial rotatory dispersion, as the mean value for the unheated serum is 0.543° and for the heated serum 0.689°, a difference of 0.146°.

Discussion. The preceding results are particularly interesting when considered in connection with those condensed in the preceding chapter and dealing with the viscosity of serum as a function of temperature. This can be clearly seen in Fig. 19, where the two curves are brought together, one expressing the variations of viscosity and the other those of the rotatory power as a function of temperature.

There is a striking parallelism between the two series of experiments: above 52° there is a slight deviation in the viscosity curve. At the same

temperature a slight increase in the rotatory power can be observed. Around 55-57° viscosity stops decreasing. At the same temperature the rotatory power clearly begins to increase. At 58° viscosity increases, and around 61-62° the increase becomes very rapid. The increase of the rotatory power follows the same upward progression. But the two phenomena are slightly

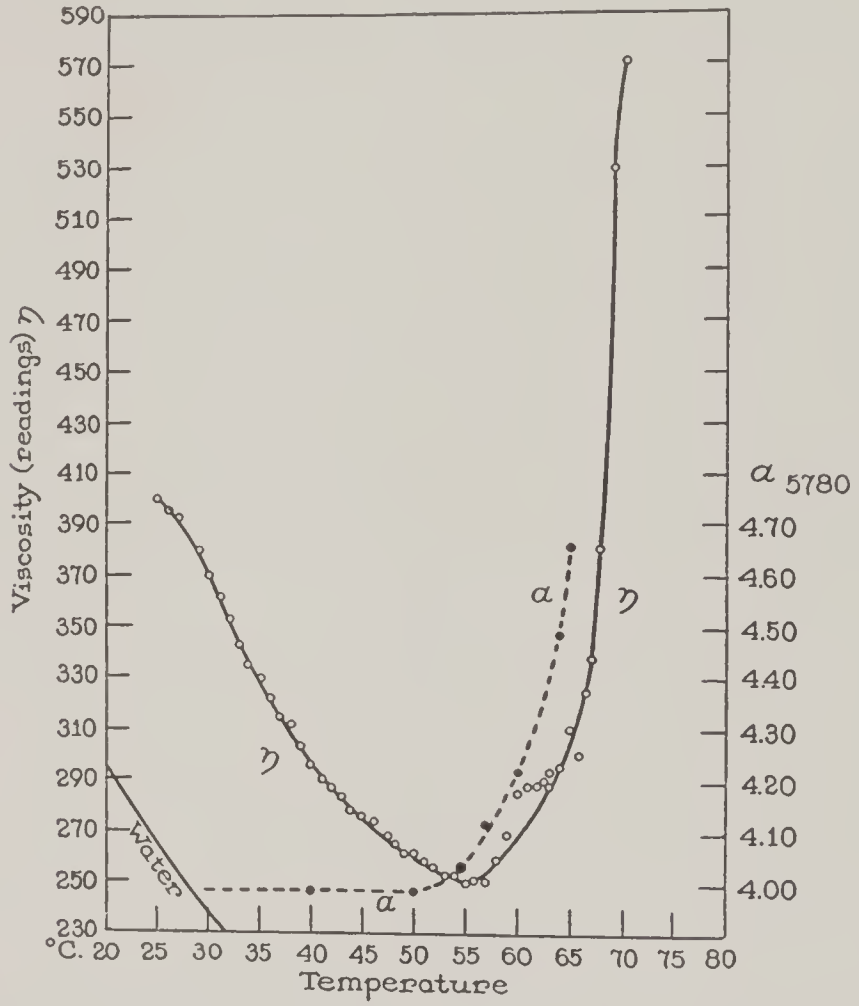

Fig. 19. Curves expressing the viscosity and the increase of the rotatory power of serum as a function of temperature.

displaced with respect to each other. The phenomenon of the rotation of the polarization plane always precedes the increase of viscosity.

Now, the viscosity curve represents one or several physico-chemical phenomena: fixation of water molecules, perhaps agglomeration of molecules in micelles,[3] etc. The polarimetric curve represents one or several chemical phenomena, that is, affecting the structure and the physical and chemical properties of the serum molecules. It can be unhesitatingly said that it is

[3] Further on we will see the reasons which led us not to admit this last mechanism as an important factor.

extremely probable that *the structural alterations revealed by the polarimeter are basically related to the modifications of viscosity*, and in particular to the existence of the absolute minimum. Moreover, they are the origin of the profound changes in the biological properties of serum.

A new optical phenomenon which manifests itself by the darkening of the fields in the polarimeter limits the readings. Everything takes place as if the angle of rotation could not increase beyond a certain limit imposed by this darkening which is perceptible only in polarized light. Practically, the increase does not exceed 0.70° to 0.80°. In one exceptional case we observed an increase of 1°. This phenomenon probably corresponds to the structural state of the serum. It does not affect the value of α and, as we have already stated, we have sometimes observed a darkening which was followed by a clearing without the reading on the dial being changed.

The problem which now confronts us is simple to state but difficult to solve. What is the nature of the structural modification introduced in the serum: does it affect the proteic part of the complex lipo-proteinic serum molecule, or another part? Does it consist in a destruction of dextro-rotatory elements or in a liberation of levo-rotatory elements? These questions are fundamentally important, for their solution would shed light on certain properties of living matter in general, for which a temperature of 56-60° is equally critical.

Chapter 5

Optical Density (Absorption) and Scattered Light As a Function of Temperature

The results obtained by our measurements of viscosity led us to admit that, as a consequence of heating, there was an increase in the volume of the molecules at the expense of the surrounding water. On the other hand, we were unable to measure any increases in specific volume, *i.e.*, hydration, greater than 327 per cent.

What happens beyond this point? There is nothing to prove that hydration continues up to complete coagulation. It was therefore of interest to verify our hypothesis by another experimental method, and to attempt to go beyond the limits imposed by the preceding one. To accomplish this we used two methods based on the estimation of the quantity of scattered and absorbed light.

It was indeed probable that, as the volume of the molecules was greatly increased, the quantity of scattered and absorbed light would increase as a function of the increase in volume. In order to try to confirm this, we measured, on the one hand, the ratio of the quantity of transmitted light, on the other hand, the ratio of the quantity of light scattered laterally, to the quantity of incident light. In other words, in the first case we measured the *opacity*, or more exactly the optical density, of serum heated between 20° and 70°, and in the second case the intensity of the light scattered at right angles by the molecules and the colloidal particles (Tyndall light), at the same temperatures.

Experimental Set-up.[1] We employed the Vernes, Bricq and Yvon photometer constructed by Jobin. This instrument is well known in France and lends itself to all sorts of problems (see Fig. 30). The experiments made by the designers showed that it was preferable to work with monochromatic light of great wave length. For this reason, red filters are used (α and F Wratten), one of which (α) gives a practically monochromatic light, and the other a lighter shade of red, which allows the passage of a greater quantity of light but is less selective. The first filter (α) was used exclusively for measuring transmission (density). The second was reserved for

[1] Lecomte du Noüy, *Ann. Inst. Pasteur*, 44, 109 (1930).

measuring scattered light, the quantity of light scattered at right angles being much smaller than that transmitted directly. In both cases the light beam, after passing through the liquid, or after its scattering, is compared to a normal beam which, if necessary, has been subjected to a known decrease of intensity by means of red filters of determined density. It is evident that the amount of scattered light will increase in direct proportion to the size, opacity and number of particles in suspension. The richer the solution in colloids, the more intense is the Tyndall cone. On the contrary, the amount of light which passes through the liquid is evidently greater if the solution is clear. The *readings*, in the case of a growing opacity, give figures which vary *inversely* with the density.

The measurements were made in the following way. If, in order to realize the equality of the fields, it has been necessary to interpose in the path of the right-hand beam of light, for example (that which passes through the cell) a filter of optical density D, and in the path of the left-hand beam (that which passes through the wedge by means of which optical equality is established), a filter of optical density g, and if, moreover, the wedge has been moved to the division N, the density δ of the absorbent under observation is given by the formula:

$$\delta = 0.62 + 0.017 \times N + g - D.$$

(0.62 and 0.017 are characteristic factors of the instrument).

The densities of the different optical filters have the following values in the instrument employed:

On the left-hand beam (*measuring wedge*):

$$A_l = 1.41 \qquad B_l = 2.41$$

On the right-hand beam (*absorbent solution*):

$$A_r = 1.50 \qquad B_r = 2.49$$

This formula is applied when the dark red filter α is used.

As the coefficients of reduction, opacity, density, absorption or extinction are used interchangeably by different authors, it may not be superfluous to remind the reader of the significance of these different magnitudes.

(1) *The reduction coefficient*, expressed by r, is equal to:

$$r = \frac{I}{I_0}$$

I_0 being the intensity of the incident beam, and I that of the emergent beam. It is also called the *transmission coefficient*.

(2) *The opacity* is expressed by:

$$W = \frac{1}{r} = \frac{I_0}{I}$$

(3) *The density*, δ, is expressed by:

$$\delta = \log W = \operatorname{colog} r = \log \frac{I_0}{I}$$

(4) *The absorption coefficient* is defined by

$$I = I_0 e^{-Kl}$$

l being the thickness of the absorbent in centimeters. All the cells employed being 1 centimeter thick, we have (M being the classical modulus 0.434):

$$\delta = \log \frac{I_0}{I} = MK \text{ and } K = \frac{1}{M}\delta, \left(\frac{1}{M} = 2.30\right)$$

The absorption coefficient is, therefore, nothing but the density multiplied by the constant factor 2.30.

(5) *The coefficient of extinction*, mostly employed in England and America, is numerically equal to the reciprocal of the thickness capable of reducing the intensity of light to 1/10 of its value. It has been demonstrated that this coefficient is expressed by the negative logarithm of the intensity of transmitted light, when the thickness of the cell is 1 centimeter.

To express the results of our measurements we shall use the density exclusively. This quantity has the advantage of being easy to handle and of having a clear significance. Indeed, an absorbent of density 2 has an opacity of 100 and a reduction coefficient of 1/100. This means that only 1/100th of the incident light can pass. An absorbent having a density of 3 has an opacity of 1,000, and so on.

It must be remembered that δ is a logarithm, which means that a value of $\delta = 1{,}602$, for example, expresses a density *double* that of $\delta = 1{,}301$.[2] This notation is similar to that usually employed for pH.

Experimental

Heating of Serum. About 5 cc of serum was collected after exudation and immediately centrifuged for 5 minutes at 9,000 rpm. In general, it was kept in corked tubes in an ice-box until the next morning. Experiments made on the day of the bleeding, that is about five hours later, gave similar values. The serum was then distributed in ampules of about 5 cc, sealed with a blow-torch, and heated to the desired temperature in a large Thermos bottle filled with water and provided with an electric stirrer. The temperature was maintained constant by an immersed electric resistance. The variations during one hour of heating were not over $\pm$ 0.25°.

[2] The logarithm of 2 is equal to 0.301, and the addition of this number to a logarithm is equivalent to the multiplication by 2 of the number represented by the logarithm.

FOOD TECHNOLOGICAL RESEARCH

In Tables 11 to 14, δ and D express the values of the ratio $\log \frac{I_o}{I}$ in transmitted and in scattered light.

Table 11 and Fig. 20 give the results obtained with serum heated for five minutes at temperatures varying from 20° to 70°, and then cooled to

Table 11. (Experiment No. 2). Fresh Horse Serum Heated for Five Minutes

ABSORPTION			SCATTERING	
Filter: α Add. Filter: 0 to the left. " " A to the right.			Filter: F Add. Filter: B to the left. " " 0 to the right.	
Temp. (° C)	Reading	δ	Reading	D
20	57.5	0.10	120	5.84
40	59.5	0.13?	112	5.67?
50	57.0	0.09	120	5.84
55	57.0	0.09	121	5.85
57	57.0	0.09	125	5.94
58	57.0	0.09	125	5.94
62	58.0	0.10	100	5.44
64	60.0	0.14	86	5.16
66	63.0	0.19	74	4.94
70	"	"	Coag. 45	4.36

Table 12. (Experiment No. 3). Normal Horse Serum Heated for Ten Minutes

ABSORPTION			SCATTERING	
Filter: α Add. Filter: 0 to the left. " " A to the right.			Filter: F Add. Filter: B to the left. " " 0 to the right.	
Temp. (° C)	Reading	δ	Reading	D
Unheated (Ice-box)	57.5	0.10	126	5.95
Unheated (Room)	57.5	0.10	126	5.95
50	60.0	0.14	121	5.85
55	58.5	0.11	119	5.78
57	58.5	0.11	125	5.94
58	58.0	0.10?	125?	5.94
60	59.5	0.13	101	5.46
62	61.5	0.16	85	5.14
64	63.5	0.20	73	4.91
66	65.0	0.22	62	4.70
68			Coag. 42	4.21

room temperature. Table 12 and Fig. 21 give the results obtained by heating for 10 minutes. In the latter the measurements could not be made at 70° because the serum coagulated. The first two lines of the table give the values for a 24-hours old serum. It can be seen that, as in Table 11, the density (measured by transmitted light) begins to increase only around 60°.

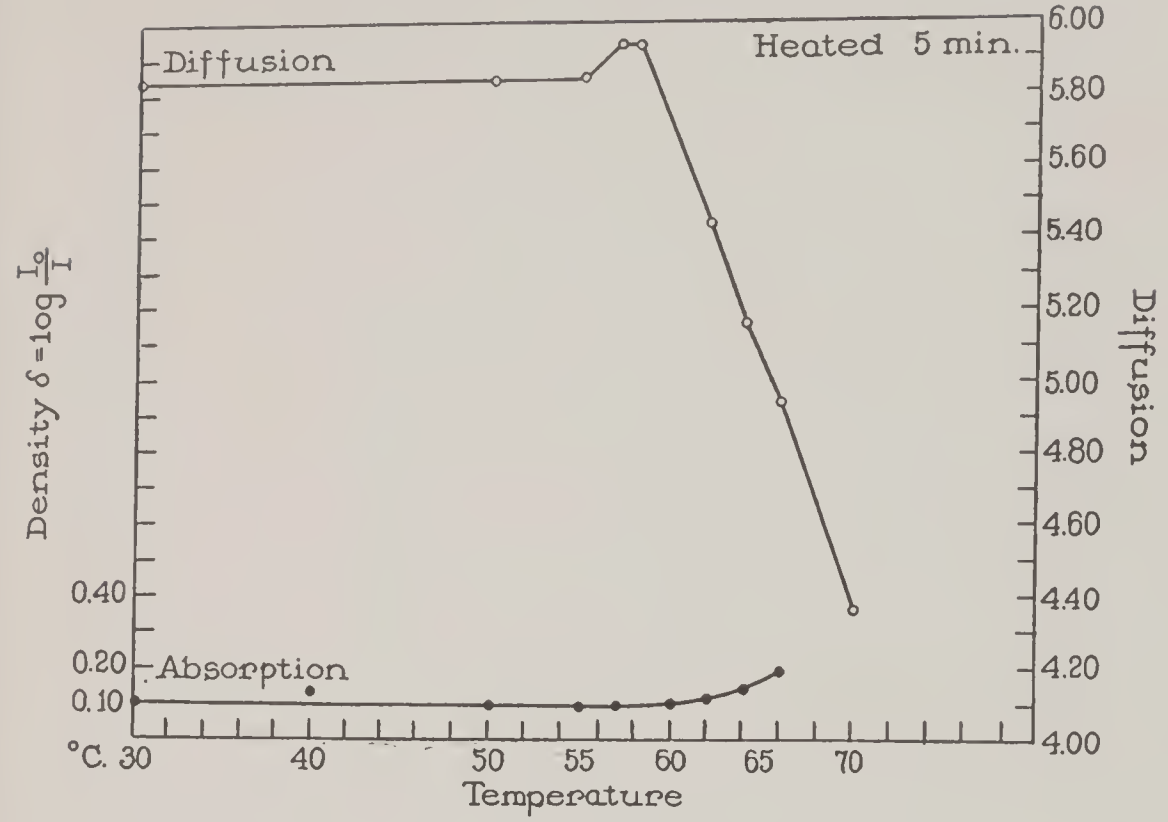

Fig. 20. Absorption and scattering of light. Normal serum heated for 5 minutes.

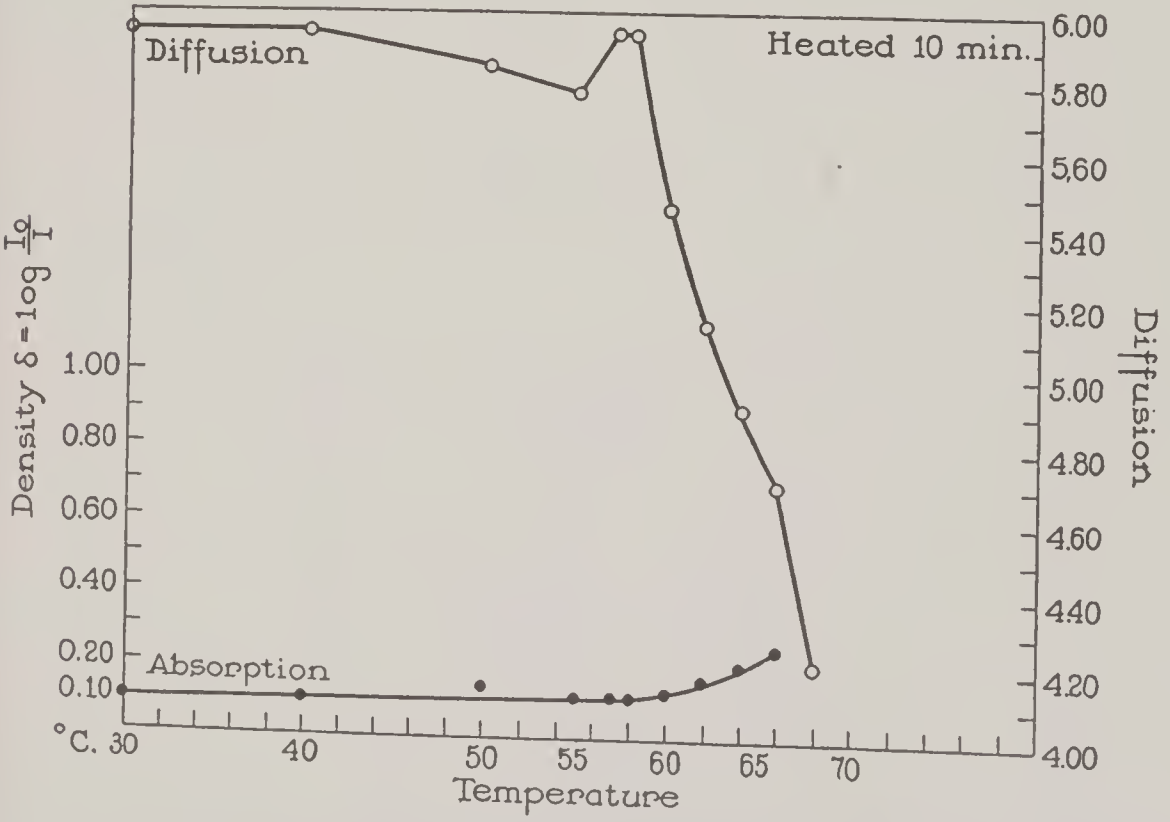

Fig. 21. Absorption and scattering of light. Normal serum heated for 10 minutes.

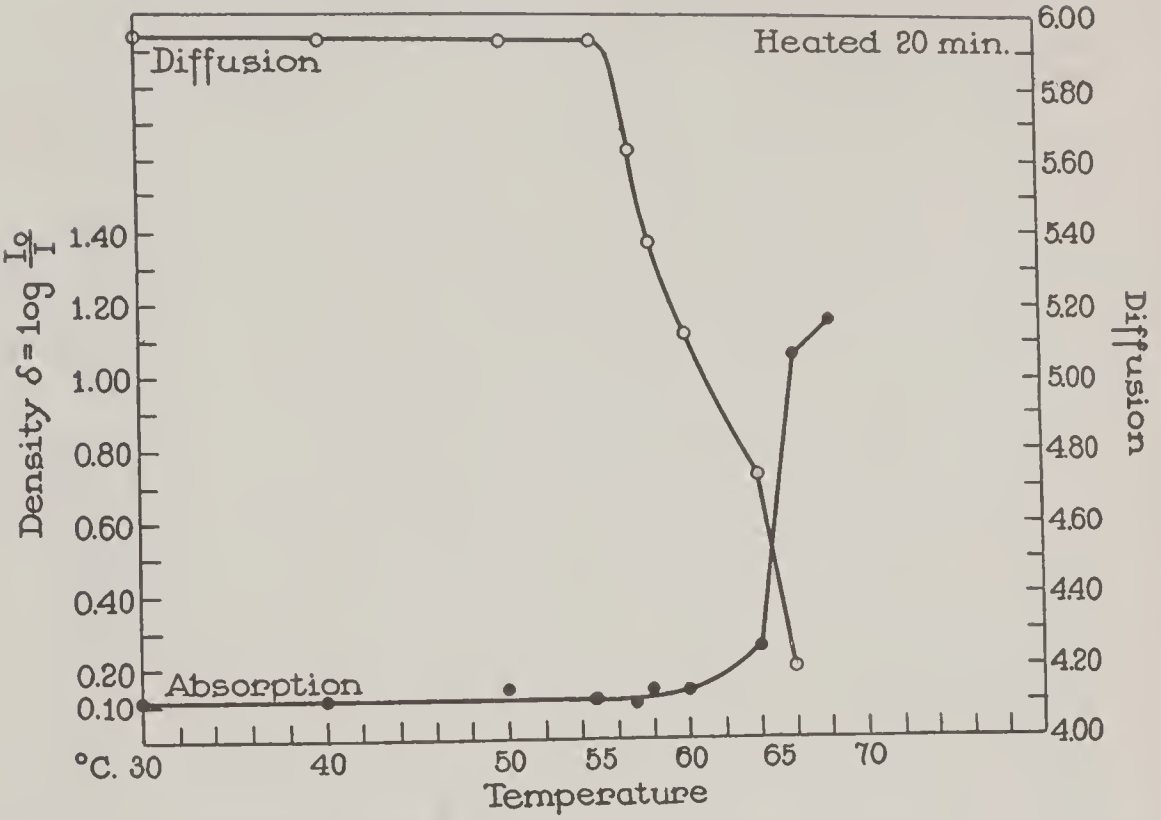

Fig. 22. Absorption and scattering of light. Normal serum heated for 20 minutes.

On the contrary, the increase in scattered light begins between 53° and 55°. Occasionally (Figs. 20 and 21), a slight decrease in the scattered light can be observed at 50° and 55°, followed by an increase which then becomes

Table 13. (Experiment No. 4). Normal Horse Serum Heated for Twenty Minutes (See Fig. 22)

ABSORPTION			SCATTERING	
Filter: α Add. Filter: 0 to the left. " " A to the right.			Filter: F Add. Filter: B to the left. " " 0 to the right.	
Temp. (° C)	Reading	δ	Reading	D
20	57.5	0.10	125	5.94
50	60.0	0.14	125	5.94
55	59.0	0.12	125	5.94
57	57.0	0.09	110	5.64
58	60.0	0.14	97	5.38
60	60.0	0.14	84	5.13
64	67.0	0.26	64	4.74
66	Coag. 115.0	1.07	37	4.21
68	120.0	1.16	39	4.25

constant. We again remind the reader that, as the lower curve represents absorption, the increase of optical density expresses increasing opacity; whereas the top curve, which is bent downward, expresses the increase of

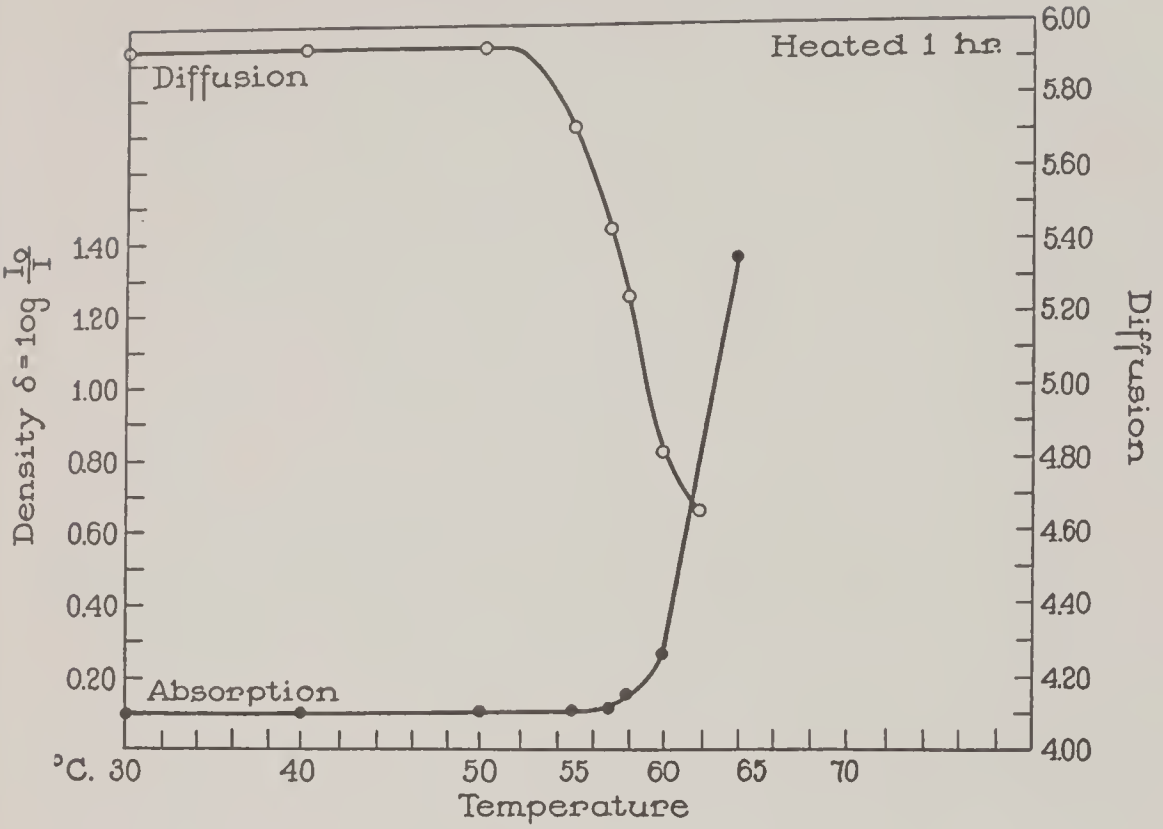

Fig. 23. Absorption and scattering of light. Normal serum heated for 1 hour.

scattered light with respect to incident light. The curves are necessarily inverted. The differences in scale must also be borne in mind. For the density (opacification) the ratio $\frac{I_o}{I}$ varies between about 1 and 20, whereas the same ratio reaches 1,000,000 for scattered light, which is always very feeble.

The preliminary decrease does not always take place (see Exp. No. 4, Table 13).

Table 14. (Experiment No. 6). Normal Horse Serum Heated for One Hour (See Fig. 23)

ABSORPTION			SCATTERING	
Filter: α Add. Filter: 0 to the left. " " A to the right.			Filter: F Add. Filter: B to the left. " " 0 to the right.	
Temp. (° C)	Reading	δ	Reading	D
20	57.5	0.10	125	5.94
50	57.5	0.10	125	5.94
55	57.5	0.10	114	5.72
57	58.0	0.10	110	5.44
58	60.5	0.15	90	5.25
60	65.5	0.23	68	4.82
62	Coag. 130.0	1.33	60	4.62

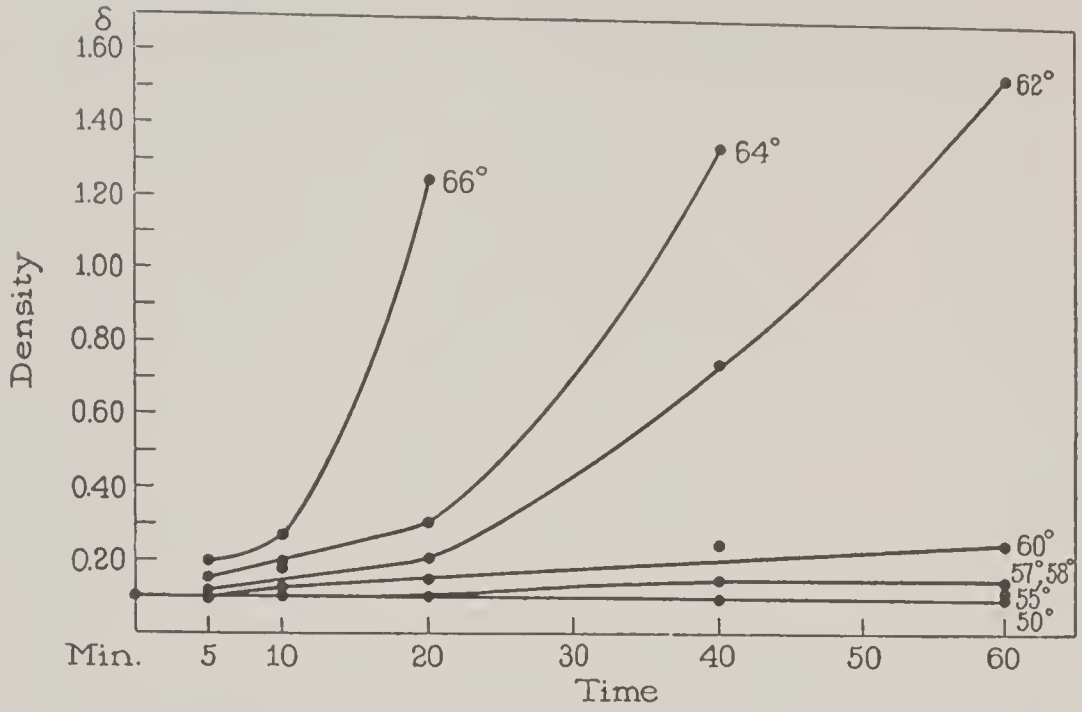

Fig. 24. Optical density of normal horse serum as a function of the time of heating at different temperatures.

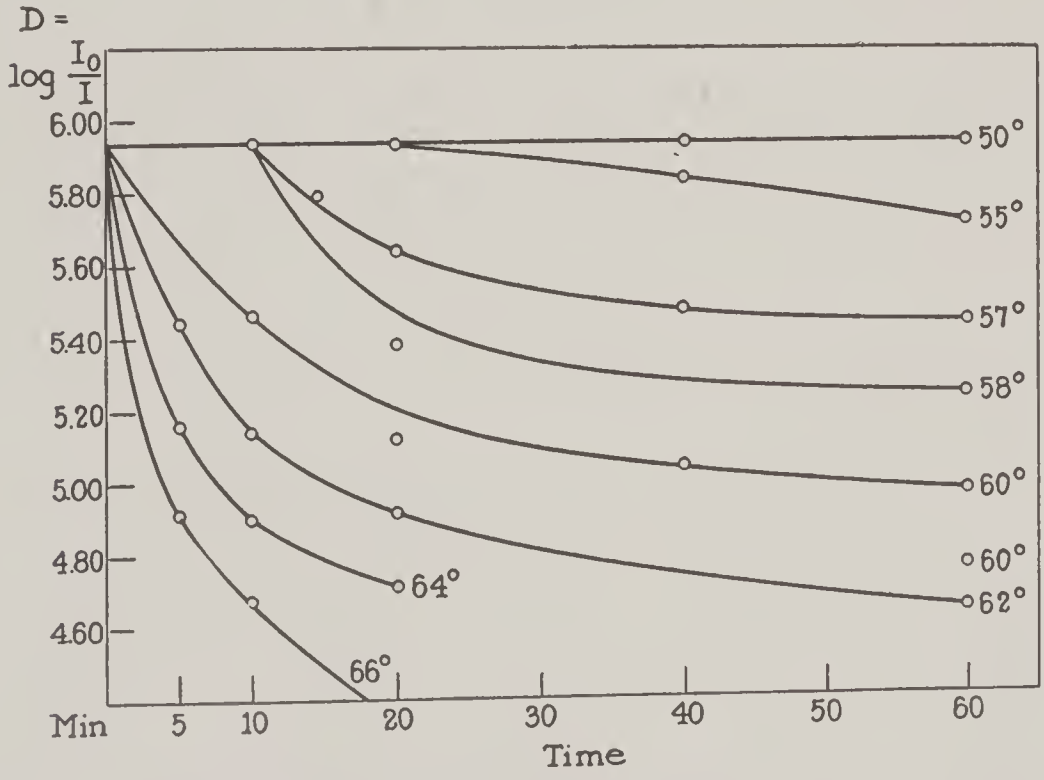

Fig. 25. Curves expressing the quantity of light scattered (90°) by the serum as a function of time at different temperatures.

There is only a slight increase below 64°, when heating is kept up for 40 or 60 minutes. The great sensitivity of the phenomenon of scattering is clearly shown by the curves.

Figs. 24 and 25, in which two complete series of experiments are summed up, clearly show the differences between the two methods. Fig. 24 (density curves as a function of temperature) indicates that up to 60° the increase is slight; but for prolonged heating the curve shows a considerable increase beyond 60°. One might almost question whether this sudden difference could not be due to a different phenomenon than the one which determines the progressive increase of density. On the contrary, no such increase is

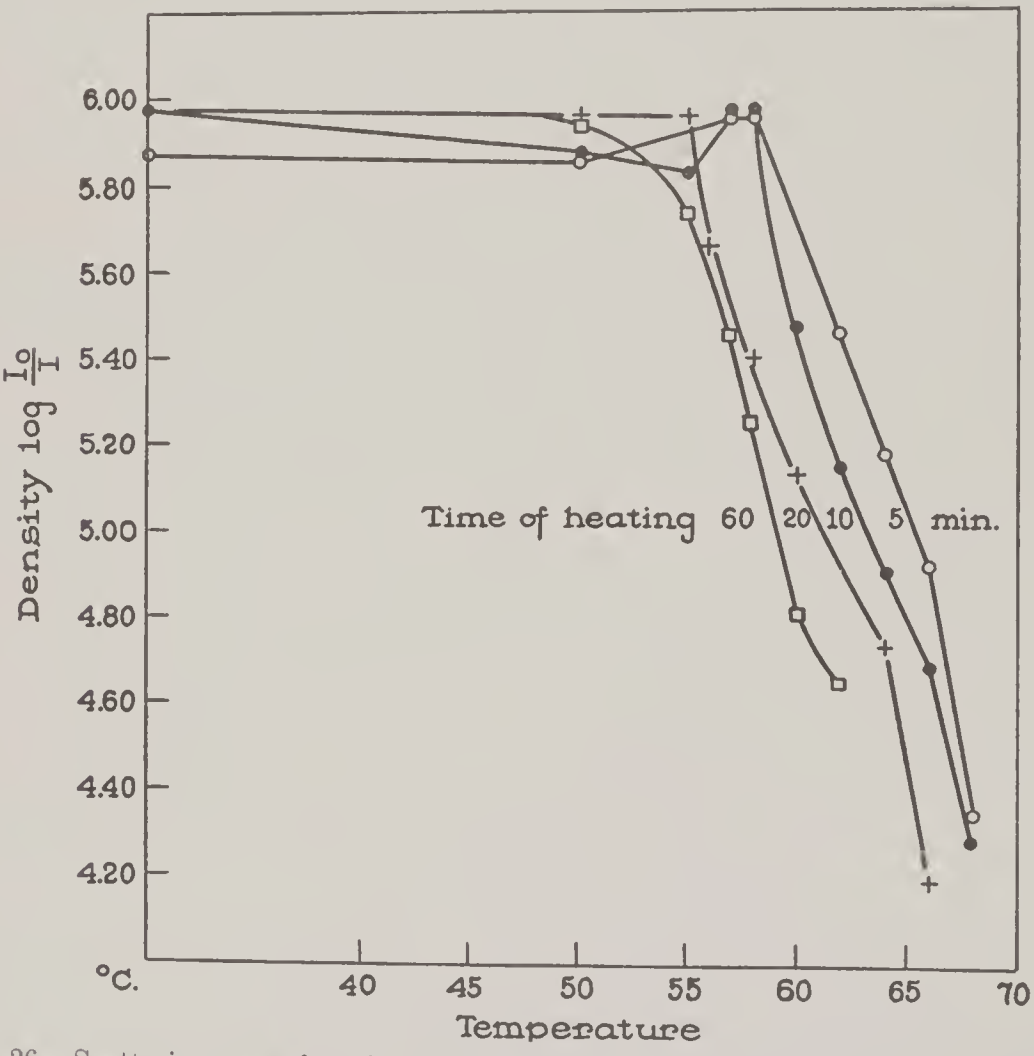

Fig. 26. Scattering as a function of temperature for different times of heating.

observed in Fig. 26 (scattering). Nevertheless, a slight acceleration in the curve representing scattered light is often observed after coagulation: the inflection starts at 66° (Fig. 21), or at 65° (Fig. 22).

The curves shown in Figs. 20 to 23 give a clear idea of the phenomenon. It is evident that the measurement of scattered light is a much more sensitive method than the measurement of density for the study of the modifications brought about in the proteins by the influence of heat, for example, and probably by many other factors.

However, we must not forget that scattering and absorption are very different phenomena which do not express, on our scale of observation, identical molecular phenomena.[3]

The clear and rapid drop of the curve, when the serum was heated for five or ten minutes beyond 57°, is remarkable. Indeed, as the figures represent logarithms, it is difficult to appreciate the amplitude of the phenomenon. But let us examine Table 13 (Fig. 22), for example, in which the serum has been heated for twenty minutes. We see that the quantity of light scattered laterally has doubled between 55° and 57°. Between 55° and 58° it has almost quadrupled. Finally, it is almost sixteen times greater (15.8) when the serum has been heated to 64° and fifty times greater for 66°.

Twenty minutes' heating at 66° determines coagulation, and the quantity of scattered light is about 54 times greater than for the same time of heating at 55°. The extreme figures show that between 55° and 64° the density had been multiplied by only 1.35. Fig. 26, which gives the comparison between the preceding experiments, shows that the influence of the duration of the heating consists simply in *displacing* the curves toward the left, as was the case in the preceding phenomena of viscosity and rotatory power.

We therefore have another phenomenon indicating a profound perturbation in serum heated around 55°-57°. We showed previously that this physical perturbation was of structural origin, and we interpreted the increase in viscosity above 58° to be the result of the increase in volume of the particles through hydration. By the experiments described in this chapter we have brought further proof of the considerable increase in the volume of the molecules or micelles formed in serum under the influence of time and heat, as up to the present we have no proof that no aggregation takes place. In spite of the sensitivity of the method, the measurements of viscosity had not shown up the phenomenon as clearly, and it was impossible to have an idea of the quantitative increase of the dimension of the particles. This now becomes possible on the basis of the following reasoning: In his important work on the Tyndall phenomenon,[4] J. W. Strutt (Lord Rayleigh) demonstrated that the intensity of scattered light is directly proportional to the square of the volume of particles, and inversely proportional to the fourth power of the wave length. The complete formula which he finally arrived at (for light scattered at right angles) is:

$$I \text{ (scattered light)} = \frac{9\nu\pi V^2 A^2}{\lambda^4 x^2}\left(\frac{n_1^2 - n^2}{n_1^2 + 2n^2}\right)^2 \qquad (1)$$

This formula can be simplified, for convenience, when measurements are

[3] See: Boutaric, A., *Rev. d'Opt.*, 2, 145 (1932).
[4] Strutt, J. W., *Phil. Mag.*, 41, 107, 274, 447 (1871); 12, 81 (1881); 47, 375 (1889).

made under well determined and constant conditions, and the following equation is obtained:

$$I = CV^2\left(\frac{n_1^2 - n^2}{n_1^2 + 2n^2}\right)^2 \qquad (2)$$

where C is a constant, V^2 the square of the volume of a particle, n_1 and n the refractive index of the particle and of the solvent.[5] Now we showed that the index of refraction of heated serum did not vary, or varied very slightly, when heated about ten minutes around 60°. But the index of the particle should decrease in proportion to the increase in volume (due to hydration). The quantity of scattered light can increase only if the first term of the second member of the equation counterbalances the second term (between parentheses).[6]

[5] See: Hatschek, E., and Humphry, *Trans. Faraday Soc.*, **20**, part 1 (1924); Krishnamurti, *Proc. Roy. Soc., A*, **122**, 76 (1929).

[6] See: Freundlich, "Colloid and Capillary Chemistry," pp. 380 ff., New York, Dutton (1926). (English Translation.)

It is probable that in the case of serum heated sufficiently to bring about an appearance of heterogeneity (milkiness) the factor between parentheses, representing the effect due to the variations in the index of refraction, which we neglected, comes into play. We have not taken it into account for two main reasons. The first is that the measurements of the index of serum heated to 70° did not show marked differences, as can be seen by the following figures:

Undiluted, Normal Horse Serum, Exp. No. 45, (Feb. 19)		
Unheated, Centrifuged Feb. 15	= 1.34910	(T = 22°)
Unheated, Centrifuged Feb. 15	= 1.34958	"
Heated:		
1 Hour at 57°	= 1.34917	"
1 Hour at 59°	= 1.34917	"
20 Minutes at 61°	= 1.34936	"
5 Minutes at 65°	= 1.34910	"
2 Minutes at 70°	= 1.34954	"

The variations affect only the fourth decimal place.

The same serum when diluted gives, under the same conditions, practically constant values (at 25 per cent, n_D = 1.33789).

The second reason is that, in order to take this factor into account, it would be necessary to determine separately the values of n_1 (refractive index of particles) which represents considerable difficulty and would introduce grave causes of error. (See below.)

It is, moreover, possible to get an idea of the order of magnitude of this factor by making the calculation with arbitrary figures. Hypothetically, it can be admitted that hydration decreases the index n_1 of the particles. The factor:

$$F = \left(\frac{n_1^2 - n^2}{n_1^2 + 2n^2}\right)^2$$

would then decrease as a result of heating.

By taking n = 1.3510 for saline solution at 17°, and an arbitrary value n_1 = 1.3700 for the particles of unheated, normal serum, we obtain:

$$F = \left(\frac{n_1^2 - n^2}{n_1^2 + 2n^2}\right)^2 = 0.000{,}088{,}36.$$

As might be expected this is what happens, and we can arbitrarily suppress this second term without introducing grave errors in the calculation, considering the relatively big increases which we observe in the value of I. It is therefore possible to compute the increase in the volume of particles as a function of temperature and of time by means of equation (2). Indeed, we know that the figures registered by the instrument give log I_o/I (the effect of filters being of course taken into account). Instead of measuring I as an absolute value, we can subtract log I_o/I from an arbitrarily chosen and constant value of log I_o, and this will give us a value proportional to log I and very close to the real value, provided the value of log I_o is plausible. By extracting the square root of the value found, or simply by finding the value of log $I/2$ in the tables, we obtain a figure which will be proportional to the volume of the particles. The value of log I_o/I for unheated serum is about 5.94, and in that case the quantity of scattered light is extremely feeble (of the order of 1/1,000,000 of the incident light). We can, therefore, take 6,000 as the arbitrary value for log I_o/I, which is equivalent to giving a value of 1,000,000 for I_o, and of the order of 1 for I. Fig. 27 gives the results of the calculations; in this diagram, the ordinates

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For heated serum, if we admit a *very* great decrease of the index n_1, *i.e.*, 1.3600, we obtain:

$$F = 0.000,019,970$$

For differences of 1 unit of the second decimal of n_1 there is, therefore, a variation of about 75 per cent of the value of F.

On the other hand, it is not absolutely certain that the hydration of one molecule, under the conditions we have studied, manifests itself by a decrease in the index. We, therefore, do not know which way the correction should act. All we know is that the index of the pure total serum has a tendency to increase slightly as a result of heating (measurements made at constant temperature) and that the increase affects only the fourth decimal point. And, besides, under our experimental conditions, the increase is of the order of magnitude of the observed fluctuations when the serum is centrifuged at different times, or when it is older.

Hatschek [*Koll. Zeit.*, 48, 246 (1929)] criticizes the work of Krishnamurti (see above) and points out that the term containing the indexes cannot be neglected, at any rate for agar-agar solutions. We mentioned this at the beginning of this note. But, on the other hand, we do not think it permissible to introduce, as does Hatschek, the index of the dry substance as value for the index of the particle n_1. In a solution, the particles or molecules are normally hydrated (at nearly 120 per cent in the case of the serum proteins); it is the index of the hydrated particle which should be taken into account. However, we have no means of measuring it. It would be inadmissible to employ the indices of dry proteins of serum such as those obtained by extrapolation of D. B. Hand [*J. Biol. Chem.*, 108, 703 (1935)]. These indices do not correspond to any biological reality, and are certainly very different from those of the hydrated protein in solution. The part played by water in these large molecules is unknown, but is certainly fundamental. We observe only slight alterations in the index of total serum after heating, whereas we observe important variations of the rotatory power. Momentarily, it seems to us that Krishnamurti's conclusions, as well as our own, are the most logical and the most legitimate. Moreover, we speak only of *relative* values of increase. It would be very surprising if the tremendous variations in the value of scattered light (I) were due to the term between parentheses, when the index of refraction of total serum does not vary materially.

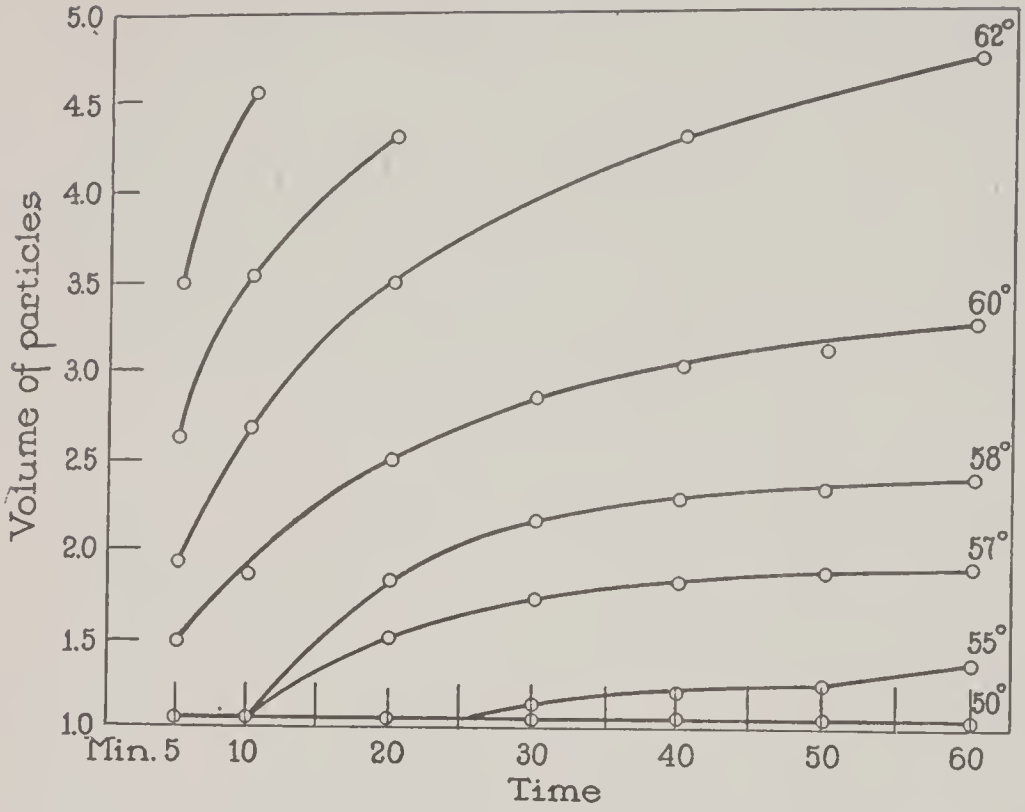

Fig. 27. Increase in the volume of colloidal particles in heated serum as a function of the time of heating.

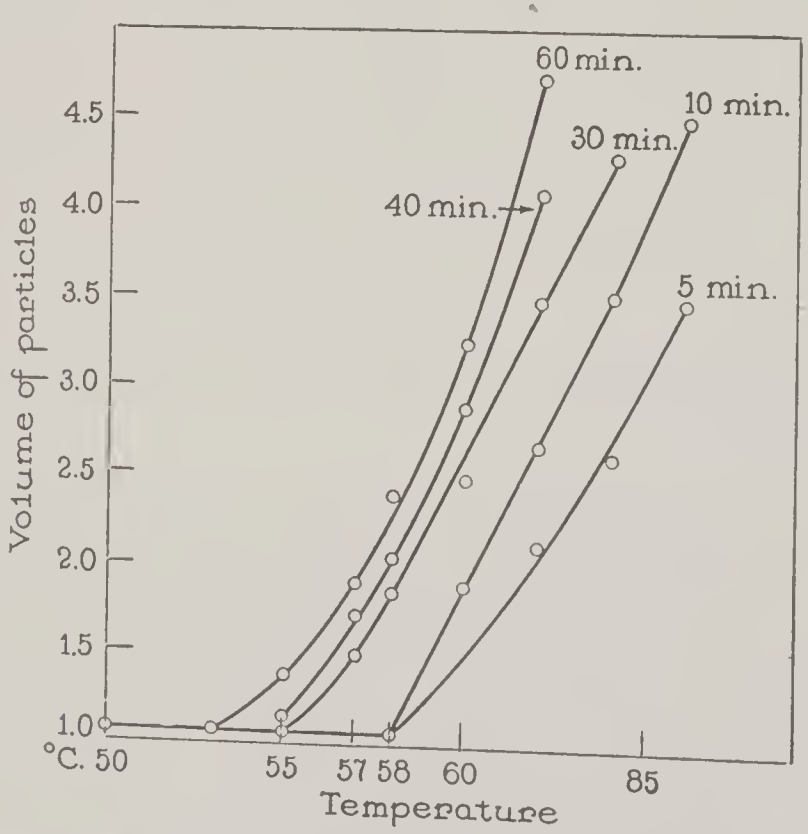

Fig. 28. Increase in the volume of colloidal particles in heated serum as a function of temperature.

are proportional to the volume of the particles. Fig. 28 represents the same values, but as a function of temperature.

The comparison between these two charts is interesting and discloses an important fact which could not be foreseen *a priori*. Indeed, it clearly shows that, above a certain temperature, the increase in the volume of the particles is about proportional to the temperature, but not to the time during which the heat is maintained. We had already observed the same fact for the increase of rotatory power, which establishes the relation between the two phenomena, and shows the specific action of temperature. Fig. 29

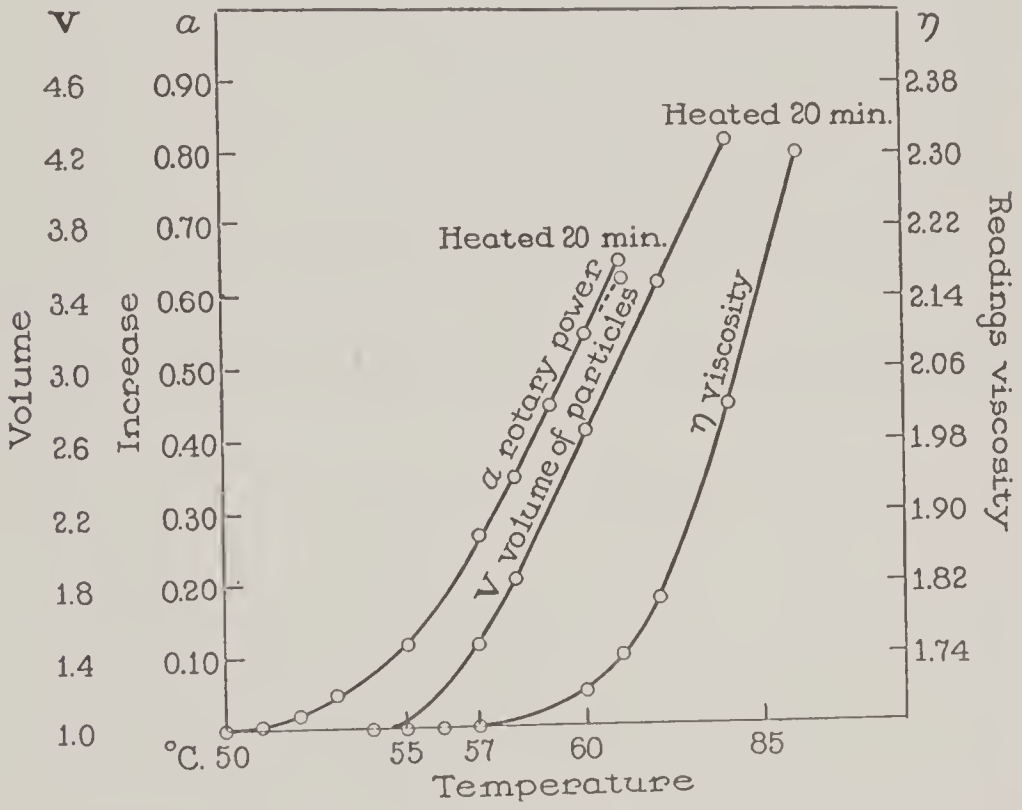

Fig. 29. Rotatory power, volume of particles and viscosity of normal horse serum as a function of temperature.

enables one to compare the three phenomena: increase in rotatory power, increase in volume of the particles, and increase of viscosity. We have, in short, only one phenomenon which manifests itself in three different ways: the alteration of the chemical structure of the molecules measured by the polarimeter naturally appears first. It is followed by the increase in the volume of the molecules or by the aggregation of the molecules into particles which scatter the light and thus enable us to follow this increase quantitatively. When the quantity of fixed water is important enough, it acts on the viscosity, with a certain delay due to the different sensitivity of the methods employed.

Thus, by applying the formula of Rayleigh, it becomes possible through the study of scattered light to extend the results obtained by the measurement of viscosity, and to observe probable increases of the order of 500 and even, as we shall see in the next chapter, of 600 per cent. As the serum molecules are not spherical, and as Lord Rayleigh's reasoning applies rigorously only to particles having an almost spherical shape, these figures give only an order of magnitude.

Chapter 6

Factor of Depolarization; Mechanism of Coagulation by Heat

The preceding chapters show that a certain number of new facts were found which give a clearer idea of the phenomena taking place in serum at a temperature above 55°. But there are points which still remain obscure. For instance, we do not yet know whether the molecules aggregate or not, whether water is imprisoned inside the micelles thus formed, and whether, in brief, they behave like minute sponges. In other words, we do not know whether or not the serum becomes colloidal as a result of heating. The milky aspect of the heated serum seems to be an argument in favor of this hypothesis.

An interesting method can be employed to answer this question, *i.e.*, the study of the factor of depolarization ρ. This quantity is very little used and to our knowledge has never been applied to biological solutions. We shall therefore explain in a few words why we resorted to it.

In the preceding chapter we showed that the study of the light scattered at right angles enables us to follow the increase of the molecules or particles in the serum as a function of temperature, and that this method was much more sensitive than that involving measurement of the optical density of serum. Now, as we know, scattered light is polarized. Lord Rayleigh's theory explains this fact clearly, and predicts that in colloidal solutions the light should be completely polarized at 90°, if the particles are small with respect to the wave length of light, optically isotropic, and distributed at random. This theory does not apply when the particles are large and irregular. Mie,[1] arrived at certain conclusions about spherical particles (particularly colloidal suspension of gold), which can be summarized as follows. As long as the particles are small (of the order of 100 mμ, or 1,000 Angstroms), the distribution of energy follows Lord Rayleigh's formula and the beam scattered at right angles is completely and rectilinearly polarized (never elliptically). When the beam is not completely polarized, the presence of larger particles in the solution is indicated. When the particles have a diameter of 130 mμ, 90 per cent of the light is polarized. In that case,

[1] Mie, *Ann. der Physik*, **25**, 377 (1908).

there is a displacement of the direction of the maximum of polarization. Finally, Mie explains the color of gold solutions by the dimensions of the micelles.

But it has been demonstrated that these explanations did not suffice and that another element should be introduced into the calculations, namely, the shape of the particle. Gans[2] tried to solve these difficulties mathematically and, by considering only particles of small dimensions with respect to the wave length of light, he arrived at the following conclusions: (1) The aspect of the absorption curve depends largely on the shape of the particles. (2) The light scattered laterally by a colloidal solution is entirely and rectilinearly polarized when the particles are spherical; it is partially depolarized when their shape is different, and the more the shape of the particles differs from that of a sphere, the greater is the quantity of depolarized light. The measurement of the quantity of depolarized light—or of its ratio to the quantity of polarized light (factor of depolarization)—should, therefore, make it possible to obtain some data on one of the elements depending on the shape of the particles. Finally, the quantity of depolarized light also depends on the dimension of the particles. (See footnote at the end of this chapter on definitions and symbols).

On the strength of the preceding work, Krishnamurti[3] studied agar solutions and came to the conclusion that there were slight changes in the shape of the micelles passing from the sol to the gel state as a consequence of a change in temperature. The fact that the volume of the particles also plays a part in depolarization unfortunately prevents one from obtaining a quantitative idea of the phenomenon. One must, therefore, beware of too hasty conclusions.

Notwithstanding the above-mentioned experimental and theoretical works, the part played by the shape of the particles is still little known. It cannot be affirmed that the geometrical asymmetry, or if one prefers, the increase of a privileged diameter with respect to the others, systematically influences the factor of depolarization in all cases. Cabannes[4] showed that depolarization is small and remains nearly constant in the series of open-chain hydrocarbons devoid of lateral branches. In spite of the lengthening of the chain from methane to octane, the anisotropy (expressed by the factor of depolarization) varies slightly (methane $100\,\rho = 1.5$; octane, $100\,\rho = 1.8$). This means that, in this case at any rate, anisotropy is not directly related to the geometrical shape of the molecule. What is more, the work of Gans and of Mie dealt principally with metallic sols, composed of

[2] Gans. *Ann. der Physik*, 37, 881 (1912); 47, 270 (1915); 62, 331 (1920); *Zeitschr. Physik*. 17, 353 (1923); 30, 231 (1924).

[3] *Proc. Roy. Soc.*, ser. U. 122, 76 (1929).

[4] "La Diffusion Moléculaire de la Lumière," p. 135, Paris, 1929.

opaque particles. Their results cannot be generalized to particles such as those of proteins endowed with totally different properties in respect to light.

In any case, even if it is impossible to obtain information on the geometry of the molecule by the sole determination of ρ, one can at least get some direct data on molecular anisotropy. And this can be done without having to resort to the orientation of the molecules by an auxiliary electric or magnetic field, as Cabannes points out.[5]

The important fact to remember is that ultramicroscopic particles are generally spherical and isotropic, and that they therefore decrease the value of ρ. *A solution which scatters almost completely polarized light is a colloidal solution and not a homogeneous liquid.*[6]

Now, we have already expressed quantitatively the increase in volume of the serum particles as a function of temperature and of the time of heating. We therefore thought that it might be interesting to study the factor of depolarization in the hope that some precise information might be gained about the way in which the increase in volume of the serum particles takes place, first through hydration and then, perhaps, through aggregation with other molecules, according to the most generally accepted theory. Are the water molecules adsorbed evenly and simultaneously on all sides of the "serum molecule," so that the symmetry of the molecule remains invariable while the volume increases, or, on the contrary, do the water molecules fix themselves preferably on one or two points of the molecule, thereby changing its shape by increasing one of the axes in proportion to the others? And, in general, can we increase our still vague knowledge of the process of adsorption and get a clearer idea of the phenomenon of the coagulation of serum by heat?

It was in the hope of throwing some light on these important facts that we undertook the experiments which we shall now describe.

Experimental Set-up[7]

We employed the Vernes, Bricq and Yvon photometer, which we had already used in our preceding experiments, but it was necessary to modify it so as to adapt it to the new problems which we had in mind. (Fig. 30.)

To begin with, the luminosity of the instrument was increased eight times. Furthermore, two nicol prisms, perpendicular to each other, were placed on the path of the scattered beam and mounted on a chariot so that they could be rapidly interposed for the analysis of the light. It was decided to use two nicols fixed in an invariable position, rather than one nicol capable of rotating by 90°, in order to facilitate construction and

[5] *Op. cit.*, 107.
[6] Cabannes, *op. cit.*, 184.
[7] Lecomte du Noüy, *Ann. Inst. Pasteur*, **45**, 251 (1930).

handling. In this way the ratio of the intensity of the depolarized beam to the vertically polarized beam could be determined by measuring the values of log I_o/I after successively interposing the two nicols, without the necessity of taking into account the absorption by the two nicols, this absorption being the same in both cases. Finally, it was necessary to interpose a white scattering screen, on the path of I_o so as to be able to compare two sources of the same nature. Fig. 30 shows the set-up. It can be seen that the two objectives, D_1 and O_3, use as respective sources of light the

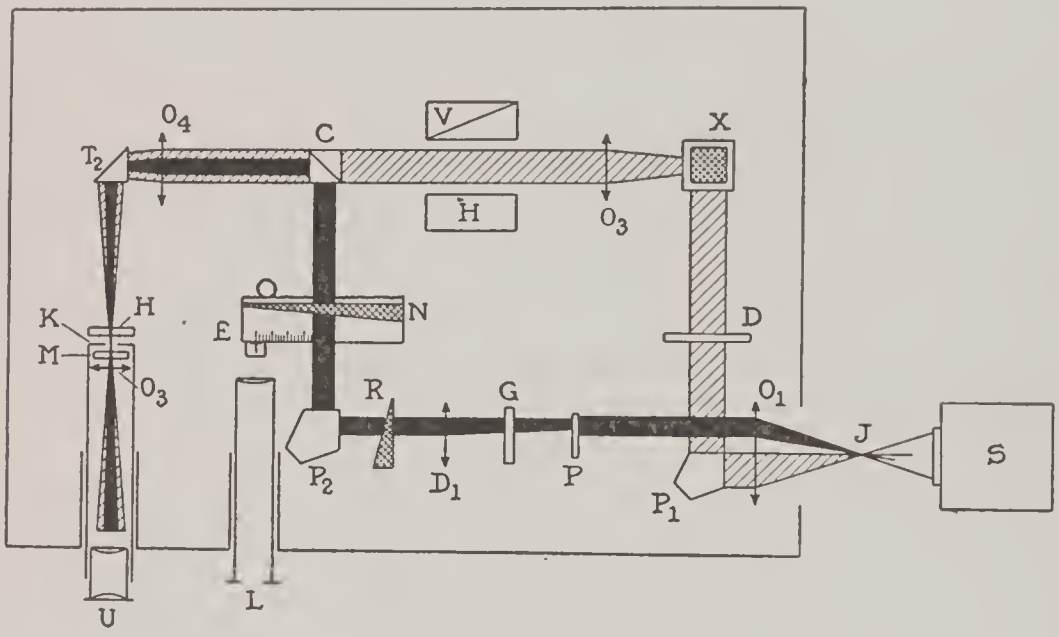

Fig. 30. View of the principal alterations introduced in the V.B.Y. Photometer. 1. A white scattering screen P was added at the focus of an objective D_1. 2. Two nicols V and H were added on the path of the beam scattered by the solution X. These nicols are mounted on a sliding chariot and can be successively put in the path of the light beam .

scattering solution X and the scattering screen P. The image is formed on the diaphragm K.

The source of light employed was generally a tungsten projection bulb of 450 watts. This intensity was required in order to be able to measure the depolarized light which is extremely weak in the case of normal serum. We also employed, in certain cases, a Philips tungsten-ribbon bulb absorbing 18 amperes at 6 volts.

Owing to the very feeble intensity of the depolarized light which represents, for serum up to 55°, about 1 per cent of the scattered light, itself only about 1/800,000 of the incident light, we were obliged to use a blue filter instead of the red filter, which is less luminous. Indeed, we know that the intensity of scattered light is proportional to the reciprocal of the fourth

power of the wave length. The following figures[8] show the importance of high-frequency radiations in scattered light:

λ (Angstroms)	2,000	4,000	6,000	8,000	10,000
Numbers proportional to λ^{-4}	635	39	7.7	2.4	1

As the red filter corresponds to a mean value of λ = 6,900 A (Wratten), whereas the blue filter No. 45-H (Wratten) corresponds to λ = 4,800 A, the increase in luminosity is obvious. The figures published here were obtained with the blue filter H (Wratten) unless otherwise specified.

The formula employed for the readings was:

$$(n + g - d) \times e = \log \frac{I_0}{I}$$

where: n = readings on the scale;
g = value of additional filters on beam I_1 (left)
d = value of additional filters on beam I_2 (right)
e = correction due to the monochromatic filter.

The value of e for the α filter is 1; for the blue filter (H), 1.27; for the red filter (F), 1.15 (λ mean = 0.675μ).

We ascertained the fact that the temperature of the liquid at the time of the reading did not appreciably influence the measurements. The same figures were obtained at 10°, 20°, and 40°.

Determination of the Factor of Depolarization

(1) Definition. I_v being the intensity of the scattered beam in a certain direction after passage through a nicol, which lets through the vertical vibrations without weakening them; and I_h being the intensity of this same beam after passage through a nicol which lets through the horizontal vibrations without weakening them: the ratio $\rho = \frac{I_h}{I_v}$ is called the factor of depolarization.

If the scattered beam is completely polarized rectilinearly in the vertical plane, $I_h = 0$; therefore, $\rho = 0$. If the scattered beam is completely depolarized, *i.e.*, made up of natural light, $I_h = I_v$; therefore, $\rho = 1$. Finally ρ is always comprised between 0 and 1, and the less vertically polarized light there is in the scattered beam, the greater its intensity. (By vertically, we mean in a plane perpendicular to the plane containing the incident beam and the scattered beam).

(2) Measurement with the Photometer. The nicol which cuts off the vertical vibrations is put into service on the path of I_2. The photometric equality is realized, Δ being the corresponding reading when the additional filters have been taken into account. The nicol which cuts off the horizontal vibrations is then put into service. The photometric equality is again

[8] Cabannes, J., *op. cit.*

Table 15. (Experiment No. 5). Normal Horse Serum. Blue Filter H.

Temp. (° C)	Time (min)	Log $\frac{I_0}{I}$ Normal Light Read	Normal Light Corr.	Vertically Polarized Light Read	Vertically Polarized Light Corr.	Depolarized Light Read	Depolarized Light Corr.	Factor of Depolarization ρ
Unheated		1.87	2.38	2.02	2.56	2.92	4.23	0.0214
	5	1.88	2.39	2.02	2.56	2.00	4.33	0.0170
	10	1.88	2.39	2.02	2.56	2.00	4.33	0.0170
55	20	1.88	2.39	2.02	2.56	2.00	4.33	0.0120
	40	1.83	2.32	2.00	2.54	2.04	4.38	0.0162
	60	1.70	2.25	1.92	2.44	1.80	4.08	0.0236
	5	1.88	2.39	2.05	2.60	1.95	4.26	0.0219
	10	1.87	2.38	2.04	2.59	1.95	4.26	0.0214
56	20	1.80	2.29	1.98	2.51	1.95	4.26	0.0178
	40	1.72	2.18	1.87	2.37	1.75	4.01	0.0230
	60	1.69	2.15	1.83	2.32	1.75	4.01	0.0204
	5	1.88	2.39	2.02	2.56	2.00	4.33	0.0170
	10	1.82	2.31	1.98	2.51	1.95	4.26	0.0178
57	20	1.73	2.20	1.88	2.39	1.84	4.14	0.0178
	40	1.60	2.03	1.78	2.26	1.67	3.91	0.0224
	60	1.60	2.03	1.77	2.25	1.68	3.92	0.0214
	5	1.88	2.39	2.04	2.59	2.02	4.35	0.0174
	10	1.81	2.30	1.95	2.48	1.90	4.20	0.0191
58	20	1.65	2.10	1.81	2.30	1.72	3.98	0.0209
	40	1.47	1.87	1.64	2.08	1.45	3.63	0.0295
	60	1.46	1.85	1.61	2.04	1.43	3.61	0.0269
Normal		1.68	2.13	1.84	2.34	1.80	4.08	0.0182
	5	1.63	2.07	1.79	2.27	1.78	4.05	0.0166
	10	1.58	2.01	1.73	2.20	1.64	3.88	0.0209
59 (dif. ser.) 18/2	20	1.46	1.85	1.62	2.06	1.34	3.50	0.0355
	40	1.31	1.66	1.47	1.87	1.12	3.21	0.0458
	60	1.23	1.56	1.40	1.78	1.90	2.93	0.0708
	5	1.63	2.07	1.80	2.28	1.70	3.95	0.0214
	10	1.51	1.92	1.67	2.12	1.48	3.67	0.0282
60	20	1.33	1.69	1.49	1.89	1.19	3.31	0.0381
	40	1.21	1.54	1.36	1.73	2.28	2.93	0.0677
	60	1.18	1.50	1.34	1.70	2.16	2.74	0.0913
	5	1.44	1.83	1.62	2.06	2.86	3.63	0.0270
62	10	1.32	1.67	1.50	1.90	2.53	3.21	0.0490
	20	1.20	1.52	1.37	1.74	2.30	2.92	0.0661
	40	0.98	1.24	1.18	1.50	1.86	2.36	0.1390
	5	1.36	1.73	1.51	1.92	2.58	3.28	0.0437
64	10	1.15	1.46	1.32	1.68	2.22	2.82	0.0725
	20	0.96	1.22	1.17	1.48	1.79	2.27	0.1290
	40	0.94	1.19	1.17	1.48	1.62	2.06	0.2640
Normal		1.74	2.21	1.89	2.40	1.85	4.15	0.0178
66	5	1.21	1.54	1.39	1.76	2.24	2.84	0.0832
Semi-Coag.	10	1.02	1.29	1.24	1.58	1.82	2.31	0.1860
Coagulated	20	0.91	1.16	1.15	1.46	1.54	1.96	0.2510
68	5	0.98	1.24	1.18	1.50	1.89	2.40	0.1000
Coagulated	10	0.90	1.14	1.15	1.46	1.46	1.85	0.4670
Coagulated	20	0.89	1.13	1.18	1.50	1.40	1.78	0.5250

realized, δ being the corresponding reading. The factor of depolarization is given by the simple formula:

$$\text{Log } \rho = -(\Delta - \delta).$$

Experimental Results.

Table 15 gives the results of a series of experiments, at different temperatures, for different times of heating. The measurements were made after cooling to about 20°.

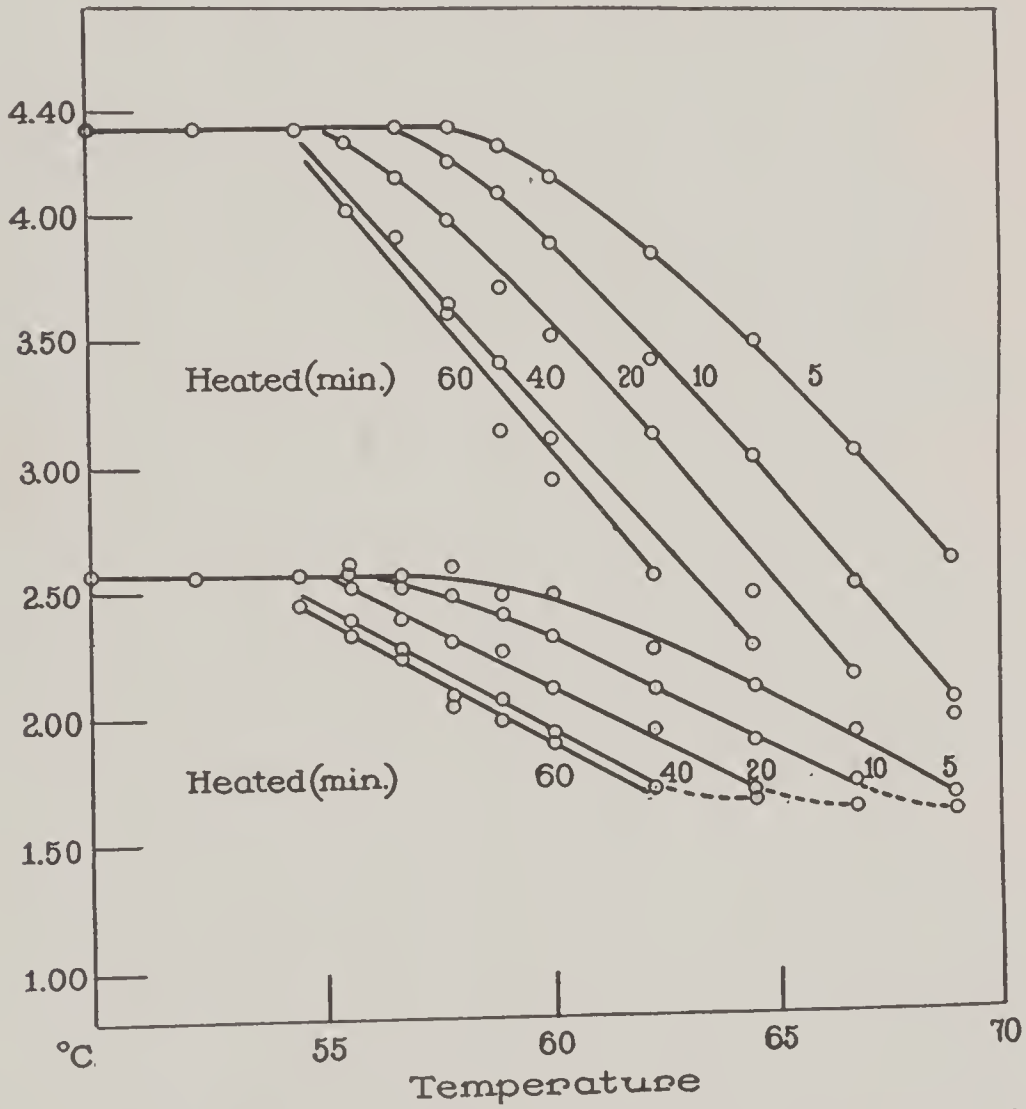

Fig. 31. Curves expressing the readings (ordinates corrected for the additional screens and for the filter H (Wratten). Depolarized and vertically polarized scattered light (lower curves) for different times of heating, from 5 to 60 minutes, as a function of temperature. Measurements made at 20° C.

When looking at this table one realizes the advantages of presenting the experimental results by means of curves, not only because of the typographical difficulties, but mostly because of the much greater clearness of

the curves. We shall therefore employ the graphic method most of the time, except when it is absolutely necessary to do otherwise.

The table shows that for unheated serum the readings were sometimes a little lower than those obtained with heated serum at 54° or 56°. As the readings express the ratio log I_o/I, this signifies that the quantity of scattered light is slightly greater than after heating. The difference is of the order of magnitude of experimental error, in general, but it sometimes corresponds to a clear phenomenon. Nevertheless, the decrease we mentioned in

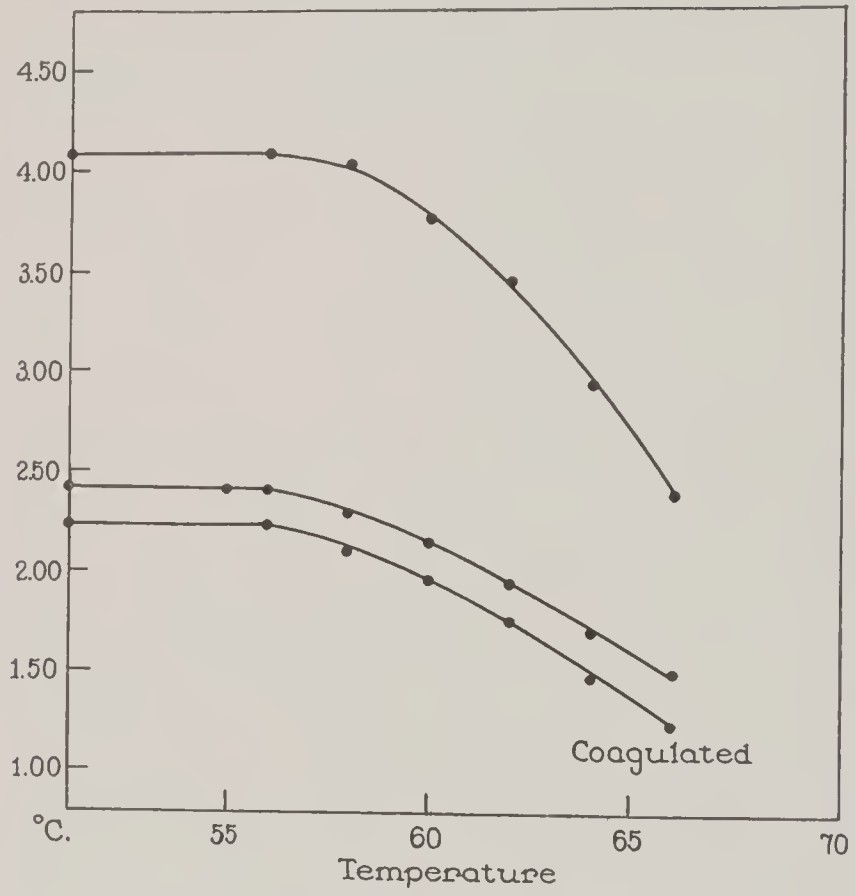

Fig. 32. Log $\frac{I_o}{I.}$. Corrected readings. Depolarized light (upper curve), vertically polarized light (middle curve), and natural light without interposition of nicol (lower curve). Serum heated 10 minutes.

the preceding chapter begins at a temperature of 57°, when the serum has been heated for ten minutes, and continues regularly. We have already pointed out that when heating is prolonged (40 or 60 minutes) the phenomenon of the increase in volume of the molecules can no longer be expressed by a straight line, though it is identical in principle. *Lengthy heating, therefore, introduces a supplementary phenomenon,* a lysis or a coalescence of molecules. On the other hand, as the curves are practically parallel, there is no advantage, for the time being, in heating longer than ten minutes.

That is why we have limited ourselves in the following examples to the experiments made with serum heated for ten minutes.

The variations in the value of ρ at temperatures lower than 58° are quite important. This is due to the difficulty of measuring the ratio log I_o/I when the nicol which cuts off the vertical vibrations is used. The quantity of light which passes is extremely feeble (of the order of 1/50,000,000 of the incident light), and the equality of the fields, which are very obscure, cannot be established with precision. The readings given represent the mean values between two extremes. The fields become clear only when the serum has been

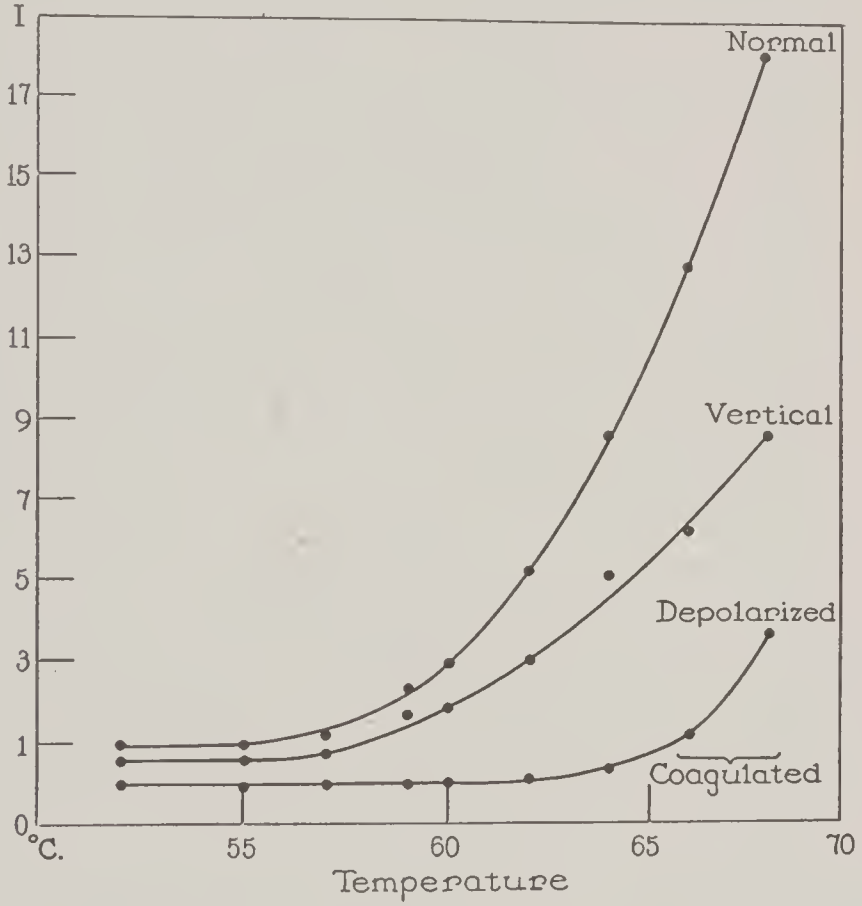

Fig. 33. Scattered light D, calculated from $n_o - n = D$, where n_o is the reading obtained for normal serum and n the successive readings as a function of temperature.

heated around 58°. Fig. 31 expresses the phenomenon for different times of heating (vertically polarized light: lower curves; and depolarized light: upper curves). Fig. 32 gives the results for 10 minutes' heating. The ordinates represent log I_o/I. Under our experimental conditions, absolute values may be obtained by establishing the value of the ratio log I/I_o for a given scattering screen with known additional filters, and by adding algebraically the value thus found to the reading n. For instance, for normal light, the absolute value of log I/I_o, for a normal serum (Exp. No. 9) was

-5.76; $I/I_o = 1/575{,}000$. For this same serum the absolute value of I/I_o in *depolarized* light was 1/36,000,000. These figures give an idea of the great rapidity of increase of this last ratio.

Fig. 33 shows the increase of the scattered light (normal, vertically polarized and depolarized), as a function of temperature. Fig. 34 represents these same values but on a different scale, which emphasizes the suddenness of the increase after 56°.

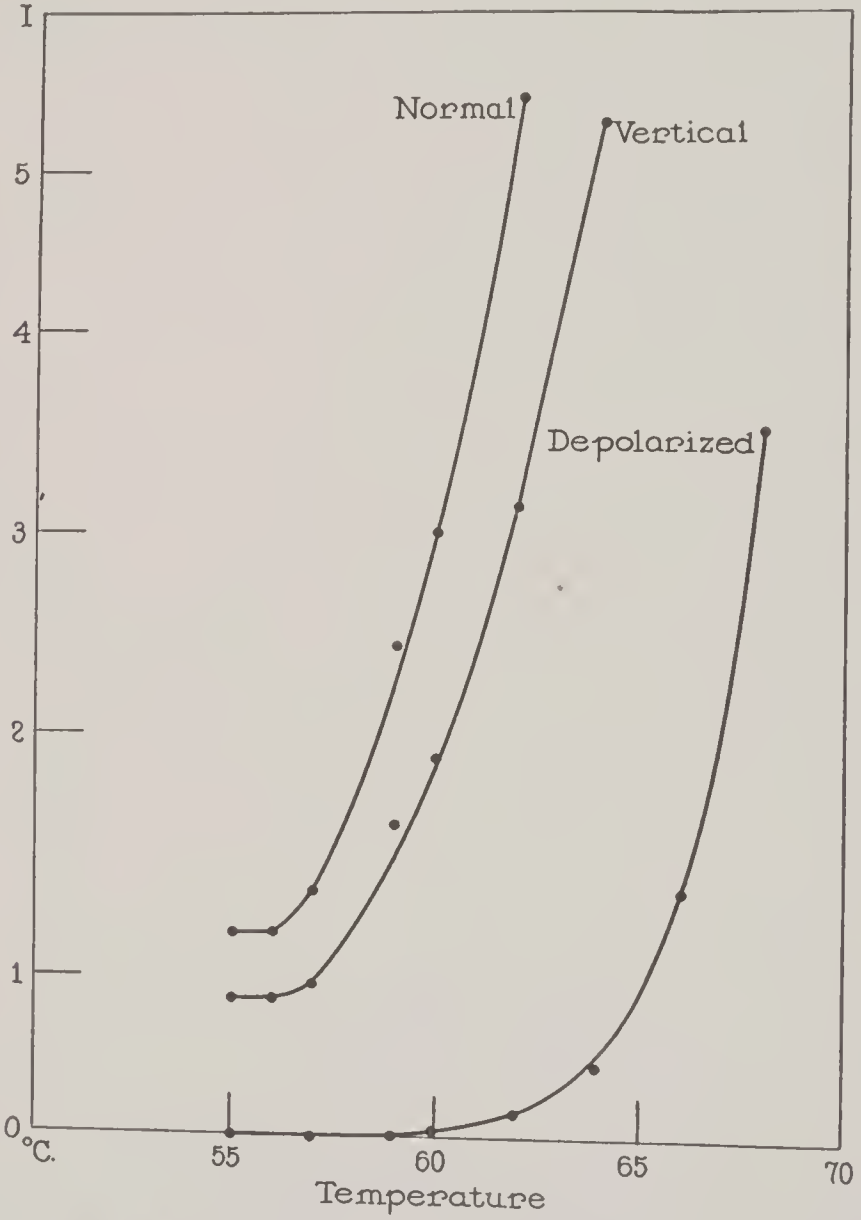

Fig. 34. Same curves as those of Fig. 33, but in which the scale of ordinates has been changed with respect to that of the abscissae so as to show the suddenness of the increase.

Figs. 35, 36, and 37 show the increase of the factor of depolarization in different experiments—Fig. 35 for different times of heating, the others for constant heating for 10 minutes. They show the high values attained by ρ in serum diluted to one-half: 0.81. This indicates that 20 per cent only of the scattered light is polarized; the optical properties of the molecules are completely changed.

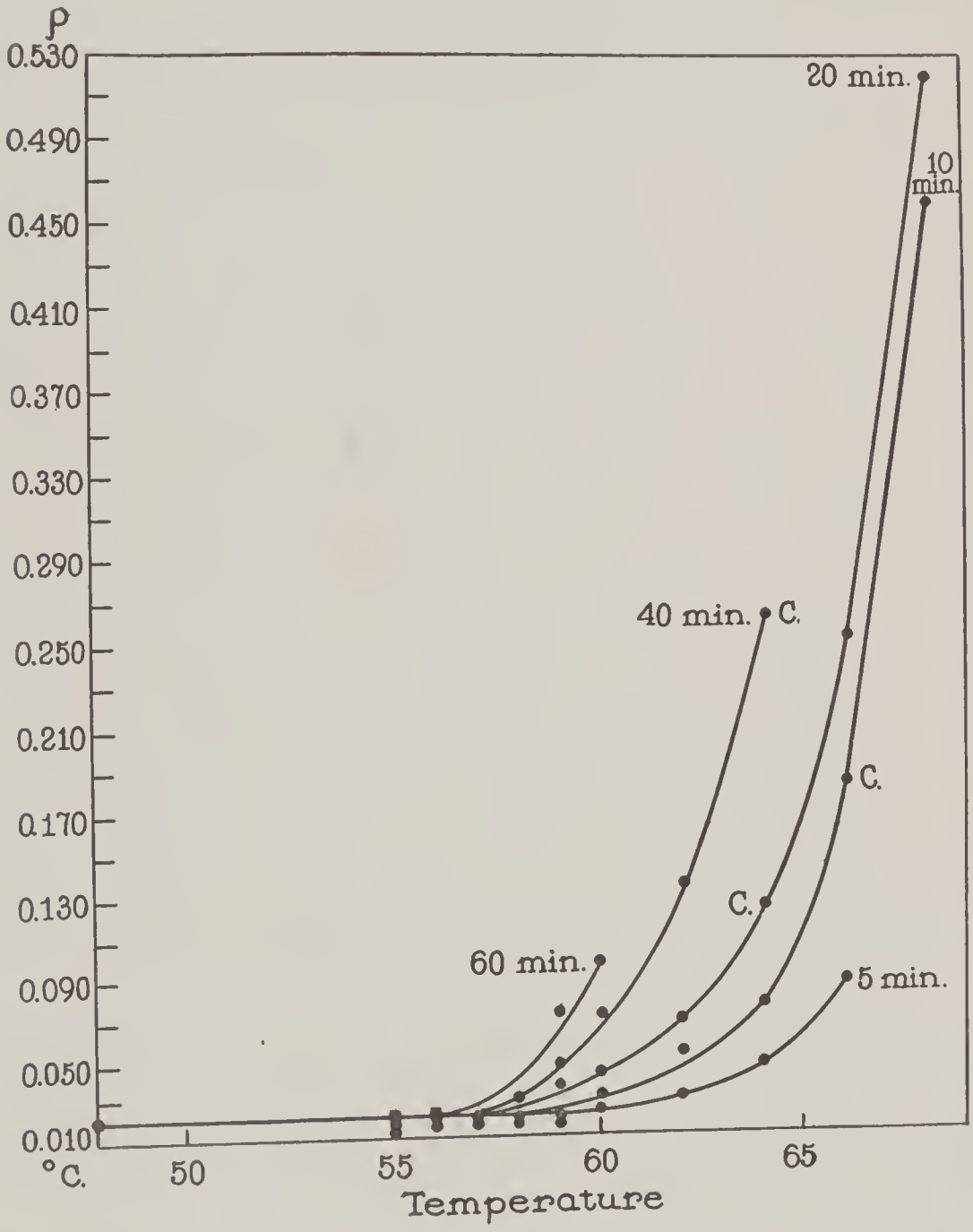

Fig. 35. Factor of depolarization. Curves expressing the increase of the factor of depolarization ρ as a function of the temperature for different times of heating. The letter C indicates coagulation.

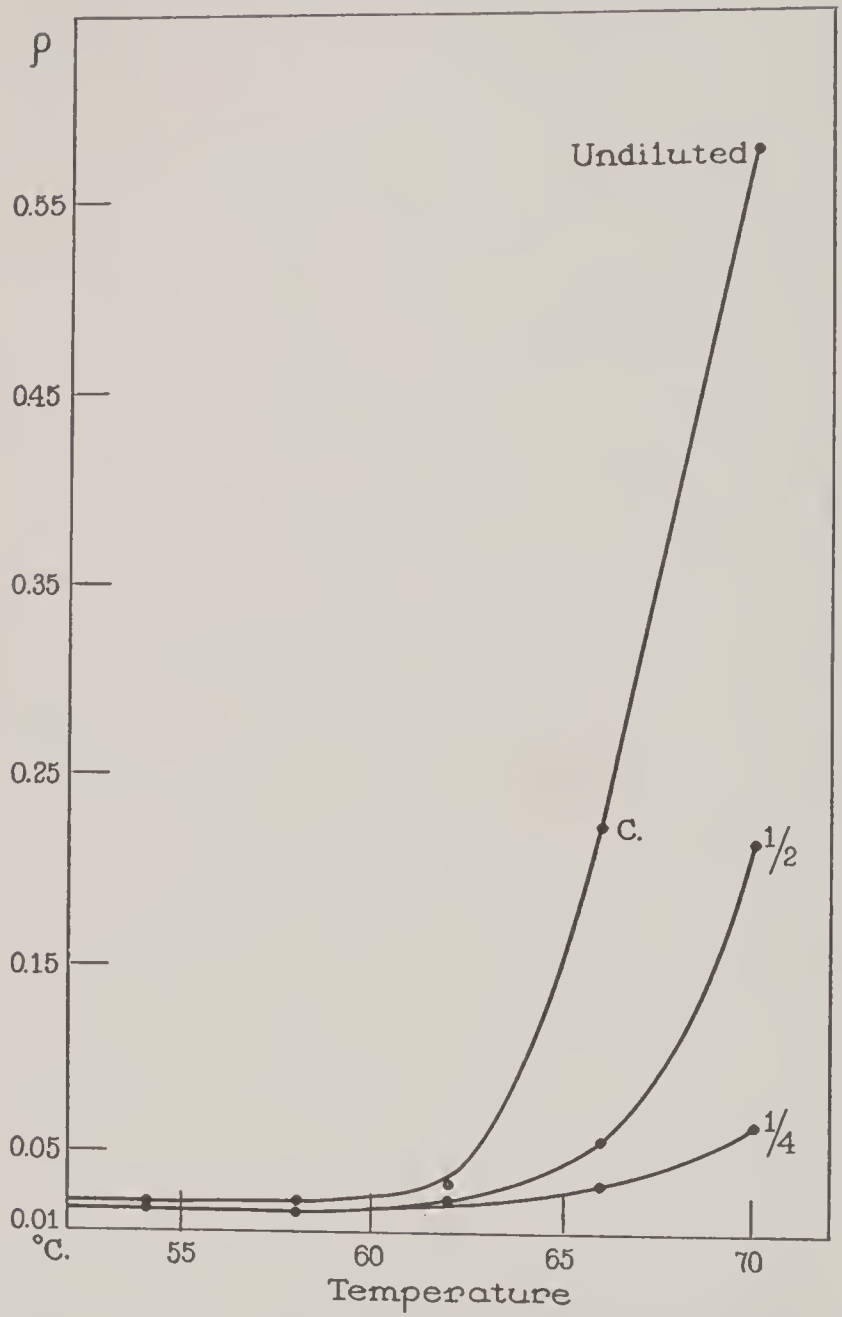

Fig. 36. Increase of the factor of depolarization as a function of the temperature for undiluted serum and serum diluted with 50 per cent and 75 per cent of physiological solution. 10 minutes heating.

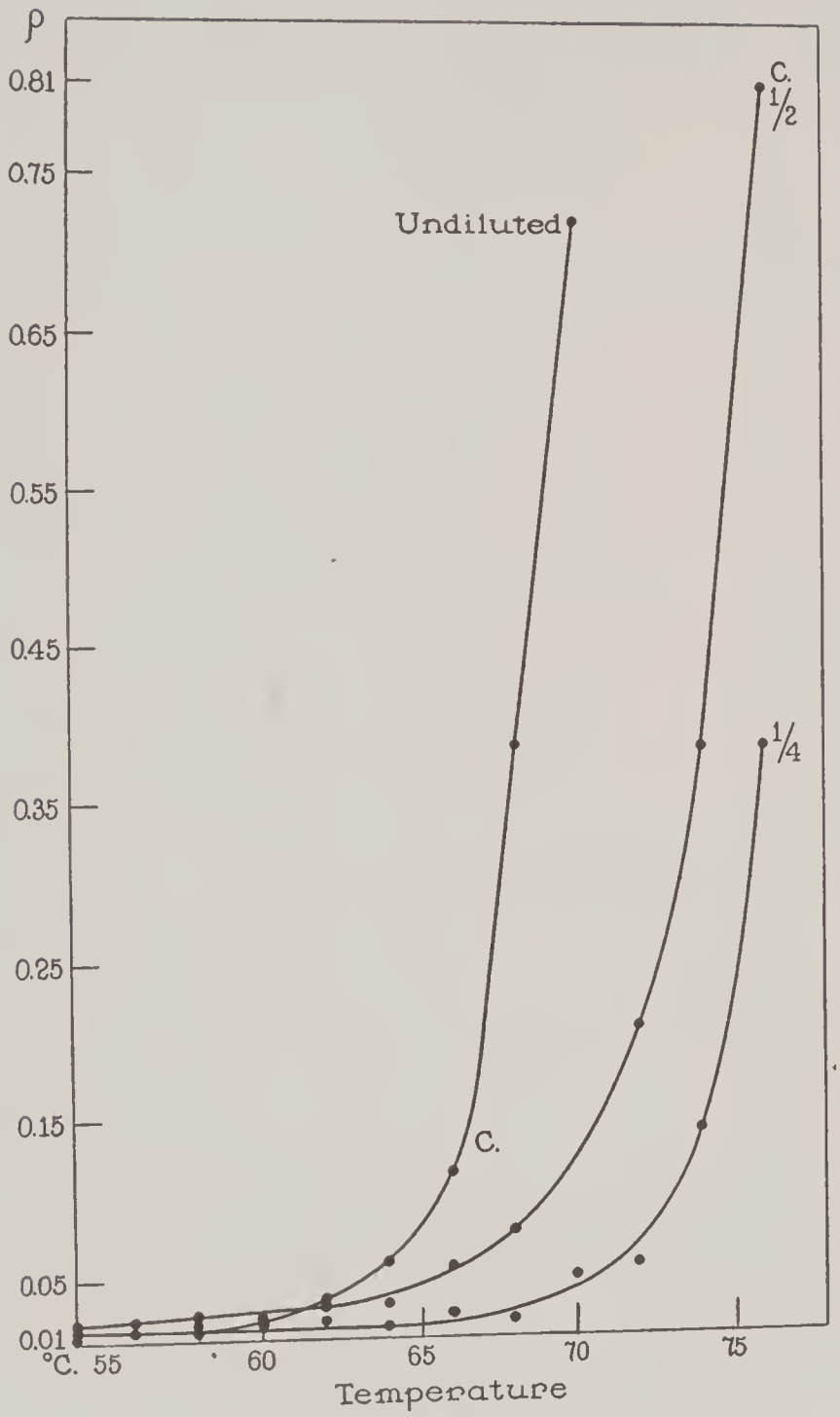

Fig. 37. Factor of depolarization as a function of temperature, undiluted and diluted serum. 10 minutes heating.

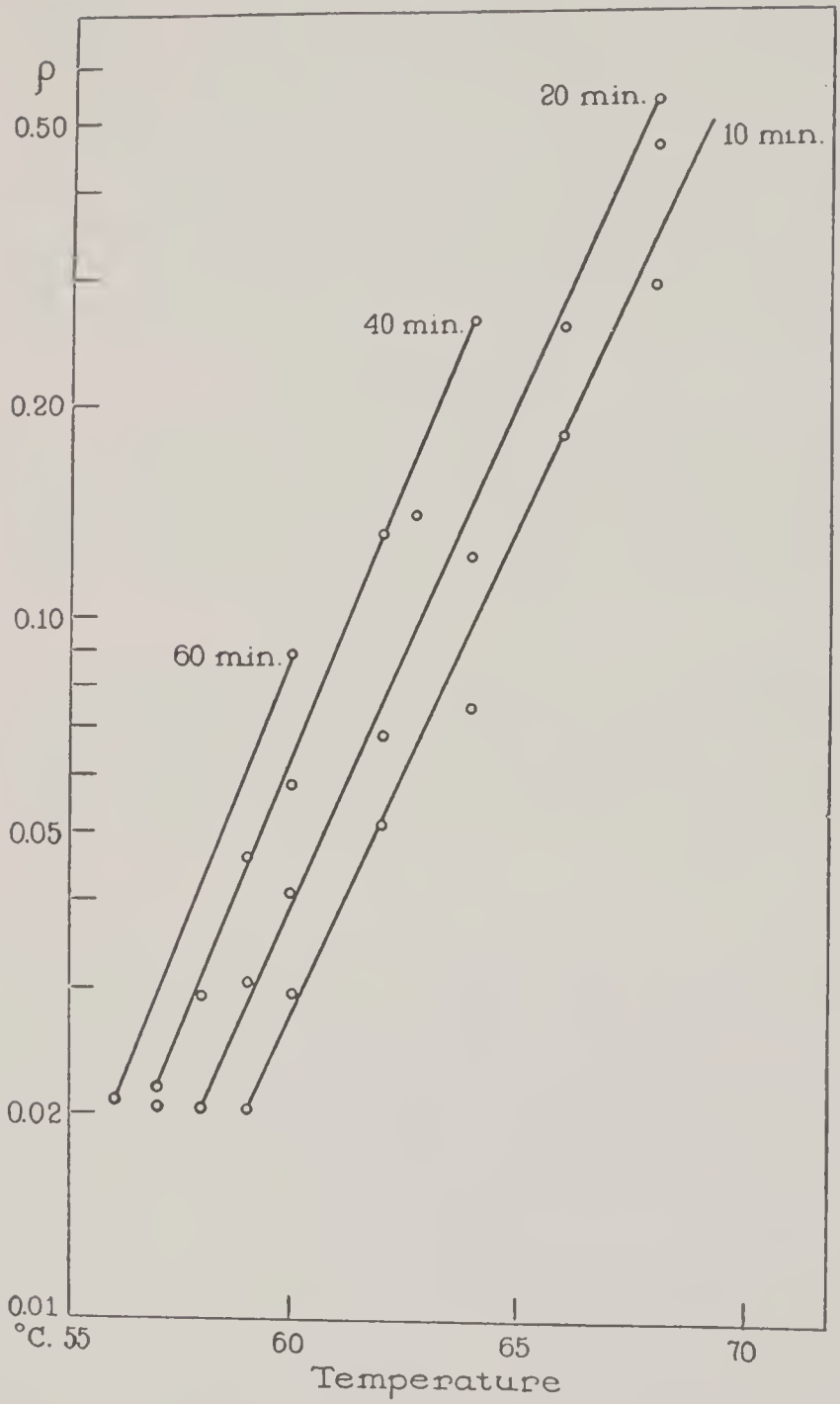

Fig. 38. Increase of the factor of depolarization as a function of the temperature, 10 minutes heating, on semi-logarithmic paper.

Table 16. (Experiment No. 13). Pure Normal Horse Serum. Constant Heating for Ten Minutes.

Temp. (° C)	Log $\frac{I_0}{I}$						Increase in Volume of One Molecule dV	Factor of Depolarization ρ
	Normal Light		Vertically Polarized Light		Depolarized Light			
	Read	Corr.	Read	Corr.	Read	Corr.		
Unheated	2.10	2.67	2.26	2.87	2.20	4.58		0.0195
54	2.12	2.69	2.26	2.87	2.20	4.58		0.0195
58	1.97	2.50	2.14	2.72	2.15	4.52	1.24	0.0160
62	1.53	1.94	1.69	2.14	1.43	3.61	2.37	0.0339
66 C	1.19	1.51	1.42	1.80	1.93	2.45	3.90	0.2240
70 C	1.05	1.33	1.36	1.73	1.55	1.97	4.79	0.5760
Same Serum Diluted $\frac{1}{2}$								
Unheated	2.14	2.72	2.30	2.92	2.27	4.67		0.0178
54	2.17	2.76	2.32	2.94	2.20	4.59		0.0224
58	2.07	2.63	2.22	2.82	2.10	4.46	1.11	0.0230
62	1.85	2.35	2.00	2.54	1.85	4.13	1.53	0.0250
66	1.40	1.78	1.57	2.00	2.55	3.24	2.95	0.0576
70	1.06	1.35	1.30	1.65	1.84	2.32	4.85	0.2140
Same Serum Diluted $\frac{1}{4}$								
Unheated	2.24	2.84	2.38	3.02	2.30	4.70		0.0209
54	2.13	2.79	2.22	2.82	2.10	4.45		0.0235
58	2.17	2.76	2.33	2.96	2.30	4.70	1.09	0.0182
62	2.00	2.54	2.14	2.72	2.00	4.33	1.41	0.0246
66	1.66	2.11	1.80	2.38	3.05	3.88	2.31	0.0317
70	1.30	1.65	1.47	1.87	2.40	3.05	3.94	0.0661

Figure 38 shows that the increases seem to follow the logarithmic law expressed by an equation of the form: $\rho = e^{Kt}$, t being the temperature.

Tables 16 and 17 represent the protocols of experiments (constant time of heating) on undiluted serum and serum diluted to $\frac{1}{2}$ and $\frac{1}{4}$ with NaCl solution at 0.90 per cent. The next to last column gives the probable increase in the volume of the molecules calculated from Rayleigh's formula. The numbers are obtained by subtracting the corrected readings (normal light) from the initial reading found for unheated, normal serum, and by dividing the difference by 2, which enables one to find the value of the increase in volume directly in the logarithmic tables.

Indeed, by calling the corrected initial reading n, and n' the corrected reading obtained after a certain time of heating, the value of $n - n'$ is equal to:

$$\log \frac{I_0}{I} - \log \frac{I_0}{I'}$$

or: $$(\log I_0 - \log I) - (\log I_0 - \log I') = \log I' - \log I, \textit{ i.e.}, \log \frac{I'}{I}.$$

Table 17. (Experiment No. 14). Normal Horse Serum No. 2, Undiluted and Diluted; Heated Ten Minutes.

Temp. (° C)	Log $\frac{I_0}{I}$ Normal Light Read	Normal Light Corr.	Vertically Polarized Light Read	Vertically Polarized Light Corr.	Depolarized Light Read	Depolarized Light Corr.	Increase in Volume of One Molecule dV	Factor of Depolarization ρ
Unheated	2.42	3.07	2.56	3.25	2.45	4.90		0.0204
54	2.38	3.02	2.54	3.22	2.45	4.90	1.06	0.0209
58	2.16	2.74	2.30	2.92	2.34	4.76	1.46	0.0145
62	1.70	2.16	1.86	2.36	3.05	3.88	2.85	0.0302
66 C	1.38	1.75	1.58	2.01	2.31	2.93	4.57	0.1180
70 C	1.18	1.50	1.45	1.84	1.70	2.16	6.10	0.4790
Same Serum Diluted $\frac{1}{2}$								
Unheated	2.26	2.87	2.43	3.08	2.35	4.77		0.0205
54	2.37	3.01	2.47	3.14	2.30	4.71		0.0270
58	2.18	2.77	2.34	2.97	2.20	4.59	1.32	0.0240
62	2.00	2.54	2.18	2.77	2.08	4.43	1.72	0.0210
66	1.68	2.13	1.83	2.32	1.62	3.85	2.75	0.0296
70	1.25	1.59	1.44	1.33	2.15	2.73	5.13	0.1260
Same Serum Diluted $\frac{1}{4}$								
Unheated	2.42	3.07	2.58	3.28	2.40	4.84		0.0276
54	2.40	3.05	2.56	3.25	2.40	4.84	1.04	0.0258
58	2.24	2.84	2.41	3.06	2.25	4.65	1.30	0.0258
62	2.15	2.73	2.34	2.98	2.25	4.65	1.48	0.0214
66	1.91	2.42	2.07	2.63	2.00	4.33	2.11	0.0200
70	1.54	1.96	1.70	2.16	1.42	3.06	3.59	0.0364

The square root of this ratio, $\sqrt{I'/I}$ is proportional to V'/V (Rayleigh's formula), *i.e.*, to the ratio of the volumes, or if one prefers, to the increases in volume of the heated particles with respect to their initial volume. This is precisely the point in which we are interested and which is set forth in the column "Increase in volume" of Tables 16, 17 and 18, as well as in Figs. 39, 40 and following. These results will be discussed at the end of this chapter.

Table 18 gives the same figures obtained with diluted serum heated at higher temperatures. The letter C placed after the figure expressing the temperature indicates that the solution was coagulated.

Figures 39, 40, 41, and 42 show the increases in volume of the molecules as a function of temperature. In Fig. 39 the times of heating are 20 and 10 minutes (undiluted serum). The appearance of a more rapid increase above 64°-65°, which we mentioned before, can be clearly seen. It is very unusual to observe this fact when the heating of the serum lasts only ten minutes, as can be noticed by looking at the following figures. This incurvation is probably due to a beginning of disintegration of the molecule and to the

variations in the value of the second term of Lord Rayleigh's formula[9] as a result of the important change in the value of the refractive indices. It is all the more marked when the heating is more prolonged.

Therefore, at first sight, if we remember the beginning of this chapter and what was said about the significance of the depolarization factor, we see that everything takes place as if, far from becoming *more* colloidal,

Table 18. (Experiment No. 12). Normal Horse Serum No. 2, Undiluted and Diluted; Heated Ten Minutes.

Temp. (° C)	Log $\frac{I_0}{I}$						Increase in Volume of One Molecule dV	Factor of Depolarization ρ
	Normal Light		Vertically Polarized Light		Depolarized Light			
	Read	Corr.	Read	Corr.	Read	Corr.		
Unheated	2.11	2.68	2.27	2.88	2.15	4.52		0.023
72	1.10	1.40	1.31	1.66	1.84	2.34	4.36	0.210
74	1.02	1.29	1.28	1.62	1.60	2.03	5.00	0.390
76 C	1.02	1.29	1.36	1.73	1.43	1.82	5.00	0.810
Same Serum Diluted $\frac{1}{4}$								
Unheated	2.13	2.70	2.29	2.91	2.10	4.46		0.023
72	1.34	1.70	1.50	1.90	2.47	3.14	3.16	0.057
74	1.12	1.42	1.31	1.66	1.97	2.50	4.36	0.145
76	0.90	1.14	1.15	1.46	1.47	1.87	5.75	0.390
Three Hrs. Heating at 90° C	1.07	1.38	1.43	1.82	1.51	1.92	4.56	0.792

serum subjected to heating becomes *less and less colloidal.* Its light-scattering elements become less and less isotropic. Its milky, colloidal, aspect is *not* the consequence of an aggregation of molecules, which would result in an increase of the statistical isotropy, but is solely due to their increase in volume by hydration, which modifies their optical properties. We shall soon give other proofs of this.

To obtain all the facts needed to follow the discussion of these results, the reader will find it necessary to refer to Fig. 27, p. 61. It shows the increases in volume of the molecules as a function of the time of heating, and each curve corresponds to a determined temperature. It may be noted that for temperatures below 60° the values tend to be constant. For two hours' heating at 55°, 57°, 58° the value is practically the same as for one hour. This tendency disappears when the serum is heated above 60°. The two unnumbered curves to the left at the top of the figure correspond to 64°

[9] See pages 58, and 59.

and 66°. The explanation of this phenomenon will be given in the last paragraphs of this chapter.

Discussion and Interpretation

Up to this point we have accepted the prevailing opinion that the micelles or molecules of serum aggregated together when heated at temperatures

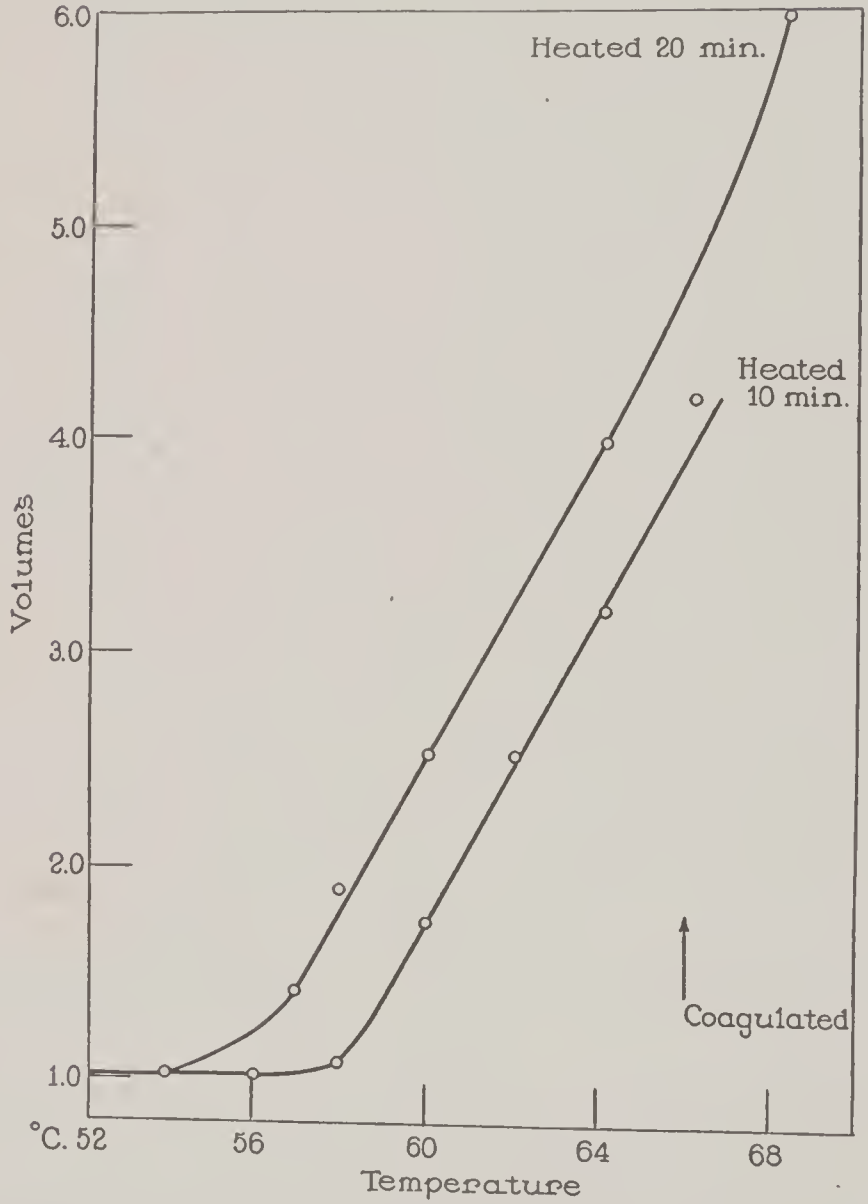

Fig. 39. Increase in the volume of the molecules as a function of the temperature. Volumes calculated by means of Lord Rayleigh's formula. The ordinates express figures proportional to the volume. Heating for 10 and 20 minutes.

higher than 56°. However, as this hypothesis does not seem to be in accord with the new experimental facts that we have just described, it may be of interest to examine anew the processes of the coagulation of serum in the

light of the observations set forth in our preceding work and especially in this chapter.

The fundamental difference between a gel and a sol consists in the fact that, as a result of some kind of modification, the substances in solution or in suspension have increased their dimensions, to the detriment of the solvent, to the point of occupying the entire volume of the solution. In

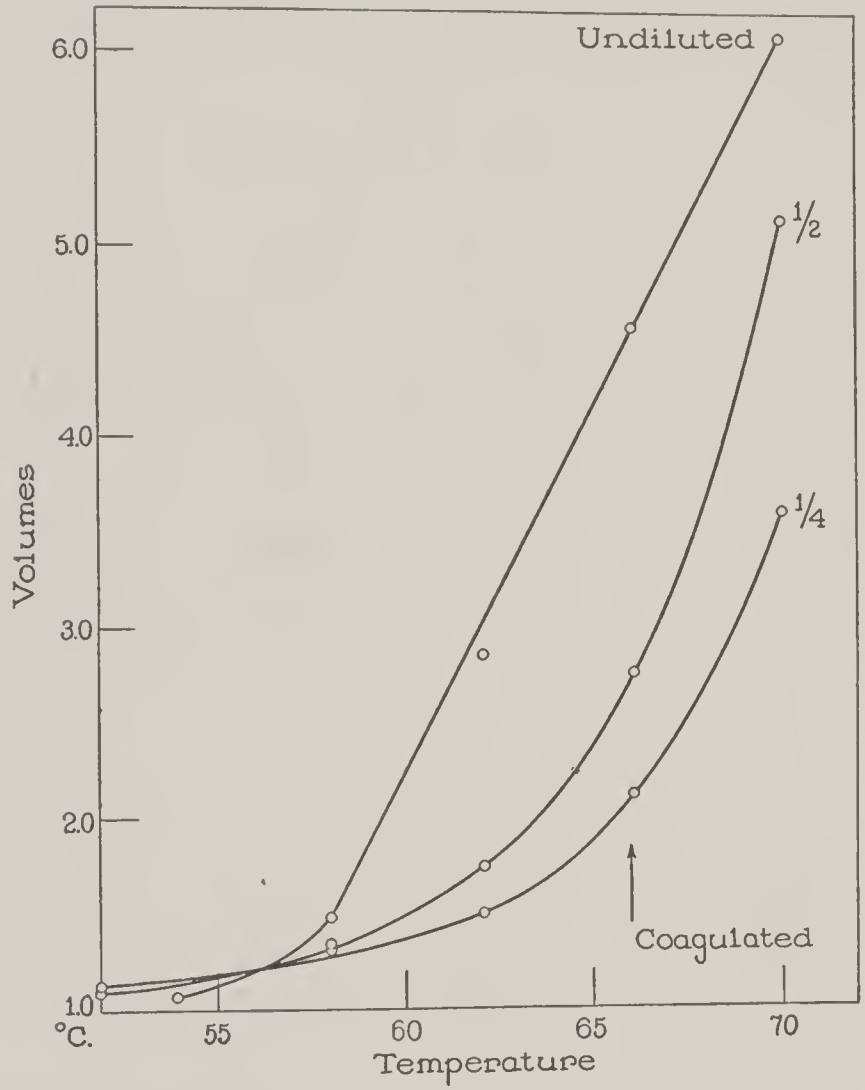

Fig. 40. Increase in the volume of the molecules as a function of temperature. Undiluted serum and serum diluted to ½ and ¼.

other words, if we call φ the volume occupied by the proteins expressed in percentage of the total volume (the mean value of which for rabbit serum is between 10.7 and 11.2,[10] and reaches 12 for horse serum), we can admit that $\varphi = 100$ for coagulated serum. The difference between φ and the real volume of the dry proteins represents the normal hydration of proteins.[11]

[10] See Chap. 1.

[11] Kunitz, M., *J. Gen. Physiol.*, 9, 715 (1926).

A given volume of normal rabbit serum, therefore, contains about 11 per cent of "serum molecules." Consequently, if the volume of each molecule is increased only 9 times, they will occupy the whole volume, in close contact with one another; the serum will become a solid coagulum and, as all the water is fixed on the molecules, there is no trace of free solvent left. *Now, this increase of the volume corresponds to an increase of only a little more than twice the linear dimensions of the molecule* (exactly $\sqrt[3]{9} = 2.08$). The "mean diameter" of the molecules would then be only doubled; that is,

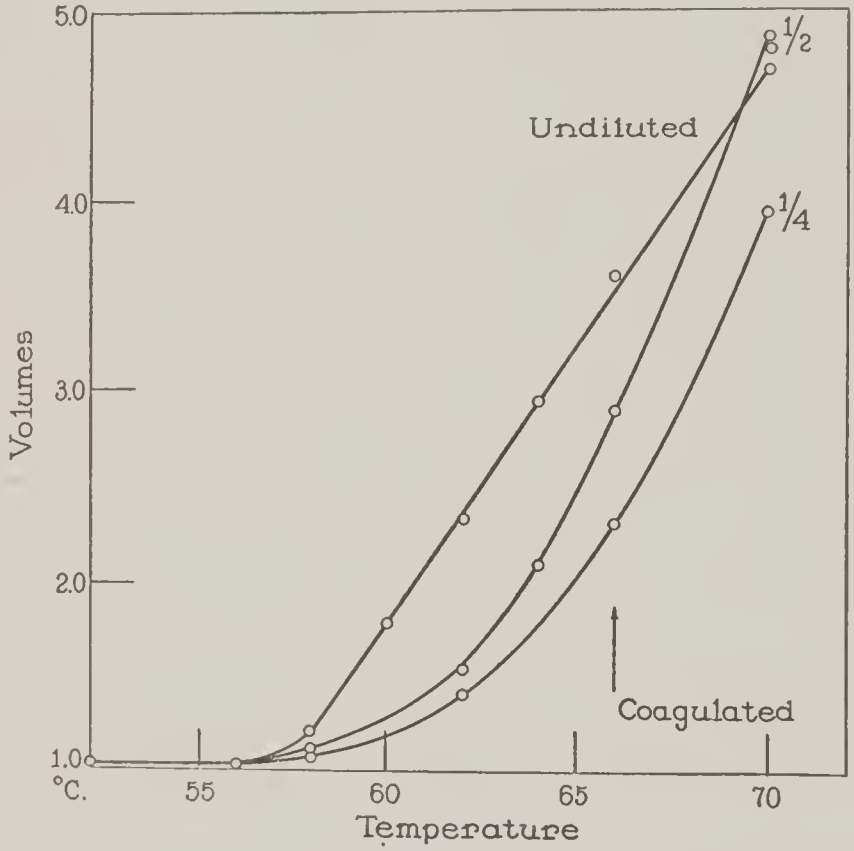

Fig. 41. Increase in the volume of the molecules as a function of temperature. Undiluted serum and serum diluted to ½ and ¼.

on the basis of some of our old determinations[12], it would be of the order of 80×10^{-8} centimeters, or 8 mμ. It would, therefore, be 50 times smaller than the shortest wave length of visible light.

But here a question arises: is it possible to admit such a degree of hydration for proteins, of the order of 900 per cent? Our measurements of viscosity gave us values of the order of 300 per cent for viscous but uncoagulated sera. Our measurements of scattered light by application of the formula of Lord Rayleigh enabled us to obtain values of 500 per cent and sometimes

[12] Lecomte du Noüy, *J. Exp. Med.*, 39, 717 (1924); *Compt. Rend. Acad. Sci.*, 178, 1904 (1924).

higher. But Marinesco,[13] by studying dielectric polarization, arrived at values of 1,100 to 1,300 per cent for ovalbumin, of 1,500 per cent for hemoglobin and of 3,600 per cent for methemoglobin. The figure 900 per cent is, therefore, quite normal for coagulated serum which is evidently highly hydrated.

The application of Rayleigh's formula led us to admit that the volume of the particles increases as much as 5 or 6 times, but only the material impossibility of making a rigid coagulum fit into the photometer cells, and the presence of disturbing air bubbles, forced us to give up the study of more

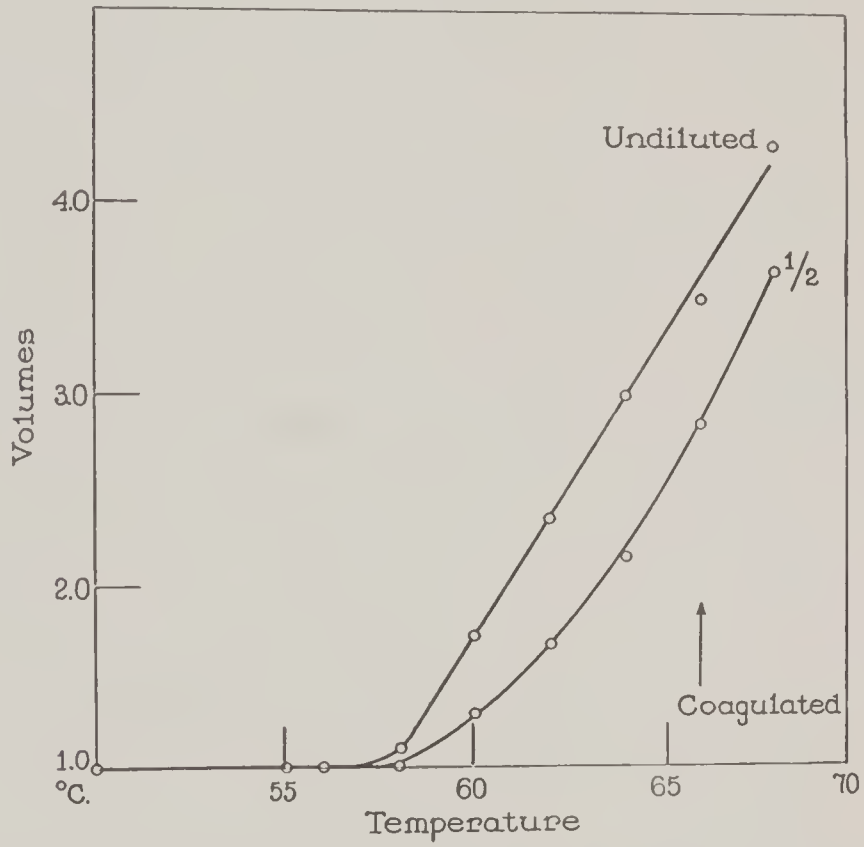

Fig. 42. Increase in the volume of the molecules as a function of temperature. Undiluted serum and serum diluted to ½.

completely coagulated sera. Now, the curve which expresses the increase of the quantity of scattered light is continuous from normal limpidity and fluidity up to complete coagulation. There is absolutely no change in the shape of the curve at the moment of gelation; the phenomenon follows the same smooth curve in spite of the macroscopic change.

Not only do the curves representing the increases in volume of the molecules show no change, as can be seen in Figs. 39 to 41, when the solution is immobilized by passing from a high point of viscosity to the stage of coagulation, but the same is true when the gel becomes more and more

[13] Marinesco, N., *Compt. Rend. Acad. Sci.*, 189, 1274 (1929); 187, 718 (1929).

rigid as a result of prolonged heating. The difference in scattered light, *i.e.*, the increase in volume of the particles, is approximately the same between two coagula obtained by heating 10 minutes at 66° and 68°, and three liquid sera heated 10 minutes at 62°, 64°, and 66°. In other words, the increase in volume, within certain limits of course, remains an almost linear function of the temperature, whether the mass is coagulated or not. This phenomenon would be very difficult to explain if the increase in volume of the micelles were due to the micelles being stuck together, whereas it becomes very clear if this increase is considered as being caused by the progressive fixation of the water molecules.[14]

There are certainly some agglomerations; but these are the exception and not the rule, and their number remains very slight in proportion to the total number of molecules. The particles seen in the ultramicroscope, and which can be observed in sugar solutions, are, as we have shown,[15] very rare in clean normal serum. Their number certainly increases as a result of heating, but never represents more than a small proportion of the molecules, of the order of 1/100,000 of the total number. This is easily checked by careful counting. These large granules probably are chiefly formed around nuclei of larger dimensions, which always exist in serum, no matter how carefully it is handled.

We thought it might be possible to get more definite information on the question of aggregation from some of the experiments described in this chapter, namely, those made with diluted serum. Let us assume, indeed, that serum is composed essentially of micelles in colloidal suspension and that the increase in volume of these micelles is the result of aggregation due to the action of heat. In this case, the number of particles which will agglomerate will evidently be proportional to the number of impacts per unit of time. In undiluted serum the number of particles per unit of volume being n, the number of impacts is proportional to n^2. If we repeat the experiments with serum diluted by half, in a 0.9 per cent NaCl physiological solution, and if we heat this solution, the conditions will be changed. There will only be one-half the number of particles per unit of volume, *i.e.*, $n/2$, and consequently the number of impacts will be proportional to $(n/2)^2$, that is to say, four times less, everything else being equal. Logically, therefore, the rapidity of increase of the particles should be 4 times less than in undiluted serum.

[14] Krishnamurti made the same observation when studying agar solutions, and drew the following conclusion: "If the increase of the diffused light were due to an aggregation of micelles, there should be a sudden increase in the scattered light at the exact moment when the sol becomes a gel, and Tyndall's number should then remain constant." *Op. cit.*, p. 89.

[15] Lecomte du Noüy, "Surface Equilibria of Biological and Organic Colloids," Chap. 10, New York, Reinhold Publishing Corp., 1926.

When the serum is diluted to $\frac{1}{4}$ (25 per cent of serum and 75 per cent of physiological solution) the phenomenon should be 16 times slower, on condition that we neglect the progressive impoverishment of the suspension as a result of the fixation of the particles. Now, we do not observe this experimentally: Figs. 40 to 42 show that even if the phenomenon is slower at the beginning, the curves later on tend to approach, and even to join, those which express the rate of increase of the particles of pure serum. Instead of being 4 or 16 times less, the rate is lower at the beginning—but never in the proportion of 1 to 4—then becomes equal and finally greater, which indicates that the increase cannot be caused by the aggregation of particles of a like nature, but by a totally different phenomenon. Now, if the rise in temperature, which leads to coagulation, does not determine the aggregation of micelles, there is no reason to admit that such amicronic aggregates exist in normal serum. The phenomenon of the increase in volume is, therefore, on the basis of our experiments, essentially one of molecular hydration in a molecularly dispersed solution.

We think we are justified in employing the word "molecule" and in eliminating the terms "particle" and "micelle," when speaking of serum in general. Serum is very likely not a colloidal solution, in the sense of a molecular aggregate, but a true solution of very large molecules, and the classical "colloidal" opaline aspect of heated serum would be caused by a molecular scattering of light. These arguments will be developed in the following pages.

In a serum diluted to 50 per cent, complete coagulation cannot take place at the same temperature for the same time of heating because, the mean distance between the molecules being doubled as their number is reduced by half, they cannot quickly attain the size which would enable them to occupy all the space in the solution. However, their volume can increase much more than that of the molecules in undiluted, heated serum, as we obtain coagulation by heating ten minutes at 76°. In this case, hydration attains a value which is evidently double, *i.e.*, 1,800 per cent, instead of 900. But this seems to be the extreme limit, for when a 25 per cent solution of serum is heated, the temperature must be brought to 90° and kept for three hours to obtain coagulation, and a new phenomenon appears: the coagulum spontaneously exudes the excess water and the concentration of the serum in the coagulum is brought back to about 50 per cent. In this case hydration amounts to about 2,000 per cent.

When the molecule is distended to the extreme, ruptures are produced in the chains, the integrity of the edifice is no longer maintained and, as a result, there are disaggregations and precipitations. The value n_1 of the refractive index of the particles is profoundly altered, and the second term between parentheses in Rayleigh's formula comes into play. One can now

understand the meaning of the well known expression "displacement of the temperature of coagulation," when the serum is diluted.

The elevation of temperature not only does not determine aggregation, but everything takes place as if, on the contrary, it led, in diluted serum, to *a fractioning of the initial molecule.* Experiments 12, 13, 14 (Tables 16 to 18 pp. 78 to 80) and the following reasoning clearly demonstrate this.

We saw (Table 16, Figs. 40 to 42) that the curves representing the increase in the volume of molecules in diluted serum were shifted toward the right (high temperatures). We have just shown how the increase of the mean distance between the molecules can by itself explain the displacement of the temperature of coagulation. But the curves expressing the phenomenon in diluted serum are not parallel to those of undiluted serum on their whole course. By assuming that our hypothesis is correct, and by applying the formula of Rayleigh, we can try to obtain an idea of the limits between which the proposed hypothesis is valid for diluted serum and of the significance of the differences.

For this let us turn back to Rayleigh's formula. For light scattered at right angles it states:

$$I = \frac{9\nu\pi V^2 A^2}{\lambda^4 x^2}\left(\frac{n_1^2 - n^2}{n_1^2 + 2n^2}\right)^2$$

where ν = the number of particles per unit of volume, V^2 = the mean square of the volume of particles, n_1 the refractive index of the particles and n the index of refraction of the solvent. The other letters represent constant quantities under given experimental conditions.[16]

By eliminating the constant values and by replacing them with another, C, this formula can be reduced as we have already pointed out, to:

$$I = CV^2$$

This is the simplified form we employed to obtain the numbers proportional to V, or more exactly to the increases of V. But if we propose to study serum diluted by half, we are forced, in order to express its behavior

[16] See preceding chapter.

The objection was made that a "gel" was considered as being formed by colloidal particles which had swollen to the point where they occupied all the available space, and that "the diffusion of a dissolved substance in this gel should be much slower than in pure water, whereas in reality it is much quicker." (Duclaux)

To this we will answer that: (1) We were careful not to assimilate the properties of "gels" to those of coagulated serum or serum in the process of coagulation, as actually no one denies the fact that serum is a molecularly dispersed solution. (2) That it happens that the diffusion of a substance such as fluorescein, for example, *is slower* in coagulated serum than in liquid serum. It is, moreover, admitted that the diffusion of large molecules can be slower. The objection, therefore, concerns only small crystalloidal molecules. But it is clear that the contact between molecules cannot be considered comparable to that of two plane surfaces. A single molecule is not assimilable to a solid body.

with respect to undiluted serum, to reintroduce the factor ν, which represents the number of particles present per unit of volume, which we had been able to eliminate as long as we were dealing with normal serum and were only interested in the changes taking place in this serum. We thus have (ν_1 denoting the number of particles in *diluted* serum)

$$I_1 = C\nu_1 V^2$$

Now as we said above (p. 79), it is not the volume of particles in absolute values which is important, but only the *ratio* of their volumes before and after heating, *i.e.*, the increase of volume due to heating. Let us call this increase, dV. As:

$$\frac{I'}{I} = k\frac{V'^2}{V^2}$$

k being a constant, this increase will be equal to:

$$dV = \sqrt{\frac{I'}{I}} = k\frac{V'}{V}$$

in the case when the *number* of particles does *not* enter into consideration.

To find out the theoretical increase of the particles in diluted serum, with respect to the increase in undiluted serum, we only have to calculate the increase of volume dV_1, with respect to dV when the number of particles ν_1 is no longer the same.

By denoting I_1, I'_1 and ν_1, the corresponding values for diluted serum, we have:

$$\frac{I'_1}{I_1} = k\frac{\nu_1}{\nu}\cdot\frac{V'^2}{V^2}$$

which gives for dV_1

$$dV_1 = \sqrt{\frac{I'_1}{I_1}} = k\sqrt{\frac{\nu_1}{\nu}}\cdot\frac{V'}{V}.$$

Indeed, if the ratios of the readings (diluted and undiluted serum)

$$\frac{I'_1}{I_1} \neq \frac{I'}{I}$$

differ, the increases in volume will evidently be the same for an identical difference of temperature, and the differences observed between

$$\frac{I'_1}{I_1} \text{ and } \frac{I'}{I}$$

will depend only on the ratio of the concentrations, that is to say, on the ratio of the number of light-scattering particles per unit of volume $\frac{}{\nu}$.

Therefore we have:

$$\frac{dV_1}{dV} = k\sqrt{\frac{\nu_1}{\nu}}$$

which means that the ratio of the increases in volume, in the case of diluted and undiluted serum, will be proportional (between certain limits of course) to the square root of the ratio of the number of scattering particles.

In the particular case where $\nu_1 = \frac{\nu}{2}$ (serum diluted by half)

$$\sqrt{\frac{\nu^1}{\nu}} = \frac{1}{\sqrt{2}}$$

therefore,

$$\frac{dV_1}{dV} = k\frac{1}{\sqrt{2}} \text{ and } dV_1 = \frac{dV}{\sqrt{2}}$$

Therefore, if there is no aggregation and if the increase is caused only by individual swelling, the increases in volume of the serum diluted by half should, on the basis of the formula of Lord Rayleigh, be proportional to the increases in volume dV in undiluted serum, divided by $\sqrt{2}$, or 1.413.[16a]

If the serum is diluted to $\frac{1}{4}$, the increases dV_2 should be:

$$dV_2 = \frac{dV}{\sqrt{4}} = \frac{dV}{2}$$

We remind the reader that the photometer we used gives, by direct reading, the values

$$n = \log\frac{I_0}{I},\ n' = \log\frac{I'_0}{I_1},\ \text{etc.}$$

wherefrom the values

$$dV = \sqrt{\frac{I'}{I}},\quad dV_1 = \frac{I'_1}{I_1}, \text{etc.},$$

are easily calculated (see p. 79).

The exactitude of this reasoning, therefore, can readily be checked experimentally.

So that, theoretically, and assuming that no other factor except individual swelling enters into play, the curves representing the values dV, dV_1 and dV_2 obtained for undiluted serum and serum diluted to $\frac{1}{2}$ and to $\frac{1}{4}$ should be easily calculated, according to our hypothesis and between certain limits, by dividing the values obtained for pure serum by 1.41 and 2.

If, on the other hand, the increase is due to aggregation of the micelles, the values of dV for undiluted serum should be divided by 4 and 16 in order

[16a] The constant k can be disregarded as we are concerned only with the slope of the curves and not with absolute values.

to express the curves corresponding to the diluted sera. The results of three series of experiments are assembled in Table 19.

It is immediately evident that the numbers in the 5th column can in no way express the phenomenon, whereas those in the 4th column follow it fairly closely up to a certain temperature. In general, there is a divergence above 66°, which can become very great (Experiments 1 and 3). Even the appearance of the curves obtained with diluted serum indicates that there cannot be an absolute accord, as those which correspond to undiluted serum

Table 19.

Ten Minutes Heating at ° C	Pure Serum dV Obs.	Serum Diluted $\frac{1}{2}$ dV_1 Obs.	Values Calculated from $dV_1 = \frac{dV}{\sqrt{2}}$ No Aggregation	Values Calculated from $dV_1 = \frac{dV}{4}$ Aggregation
		Experiment 1 (March 20)		
58	1.32	1.00	0.95	0.33
60	1.75	1.28	1.24	0.44
62	2.30	1.65	1.63	0.57
64	2.95	2.10	2.10	0.74
66	3.55	2.80	2.52	0.89
68	4.28	3.60	3.04	1.07
		Experiment 2 (March 26)		
58	1.20	1.05	0.85	0.30
60	1.45	1.15	1.03	0.36
62	2.25	1.60	1.60	0.56
64	3.20	2.50	2.26	0.80
66	4.17	2.93	2.95	1.04
68	5.20	3.60	3.60	1.28
		Experiment 3 (March 31)		
58	1.20	1.10	0.85	0.30
60	1.78	1.26	1.26	0.44
62	2.32	1.53	1.64	0.58
64	2.94	2.09	2.08	0.73
66	3.60	2.89	2.56	0.90
70	4.68	4.80	3.31	1.17

are nearly rectilinear and the others show a strong incurvation. When the serum is diluted to $\frac{1}{4}$ the accord is sometimes as good as in the above experiments, but this is not always the case. Nevertheless, and this is the only point upon which we insist, the numbers *are much closer to the values observed* than those obtained when the values for undiluted serum are divided by 16. Table 20 gives the values observed and calculated for this dilution.

It is evident that other factors come into play when the serum is diluted and heated. But from 66° on, all the curves of diluted serum tend to join

or even to cross the curve of undiluted serum, that is to say, the quantity of scattered light increases more rapidly and is sometimes greater in diluted serum than in undiluted. On the other hand we know that there are no aggregations. Only one variable capable of affecting the quantity of scattered light remains, namely *the number of scattering particles*. If the concentration stays constant, this number can increase only if we assume that there is a fractioning of the molecules above a certain temperature (around 65°).

The proteic structures are not indefinitely extensible, and by raising the temperature above a certain limit the molecules are destroyed and split more rapidly than when the serum is undiluted, because in this case the gel begins to form at a lower temperature. The molecules, therefore, are pressed tightly

Table 20.

Temp. (° C)	Pure Serum dV Obs.	Serum Diluted $\frac{1}{4}$ dV_2 Obs.	Values Calculated from $dV_2 = \frac{dV}{\sqrt{4}}$	Values Calculated from $dV_2 = \frac{dV}{16}$
58	1.20	1.04	0.60	0.075
60	1.45	1.10	0.72	0.091
62	2.25	1.33	1.12	0.140
64	3.20	1.62	1.60	0.200
66	4.17	1.85	2.00	0.260
68	5.20	2.65	2.55	0.320

against one another, and this impedes the breaking up of the original structure.

The measurement of the factor of depolarization for diluted serum shows that, in principle, the phenomenon is the same as for undiluted serum. The optical anisotropy becomes enormous: ρ attains the value of 0.80, which indicates that only 20 per cent of the scattered light is polarized. It is almost pure reflection. The curves are always very regular, and above 64° the value of ρ increases with great rapidity. We did not observe any transition between the liquid and the gel. When serum diluted to $\frac{1}{2}$ is heated to 76° (10 minutes) and coagulates, the value of ρ, as we have seen, is greater than that of undiluted serum heated at 70°. It is probable that the molecule is completely dislocated.

Without doubt, the conclusions which were reached, *i.e.*, that there is no aggregation as a result of heating and that serum is *not* a colloidal solution (in the sense of a suspension of amorphous and isotropic particles composed of aggregated molecules) are in contradiction to the generally accepted theory. This fact is in itself too important to be accepted without discussion

by the reader who has been accustomed for so long to the idea of a "biological colloid."

It is therefore natural that we should try to reinforce our thesis in every possible way, and that we should bring a new quantitative proof of the exactitude of our interpretation. This proof, which strikes us as very convincing, was given by Prof. A. Boutaric, whose magnificent work on colloids is well known. It is based on quite different reasoning and experiments (luminous absorption and not lateral scattering).[17]

The first part of Prof. Boutaric's paper is devoted to the mathematical study of the variations of the coefficient h in the formula expressing the intensity, I, of a beam of light after it has passed through a scattering medium of thickness l:

$$I = I_0 e^{-hl}$$

I_0 designating, according to custom, the intensity of the incident beam. This coefficient h, for small spherical particles, is given by the formula of Lord Rayleigh:

$$h = \frac{A}{\lambda^4} \nu V^2 \left(\frac{n_1^2 - n^2}{n_1^2 + 2n^2} \right)$$

where the symbols have the same significance as those of the analogous formula (for scattered light) mentioned on page 87.

Prof. Boutaric studies successively the ways in which h varies when the increase in volume of the particles is due solely to agglomeration, to individual swelling without agglomeration, or to a combination of the two mechanisms. By taking into account the fact that particles of different sizes may be present in the solution, Prof. Boutaric demonstrates that in all cases h varies proportionally to the volume V of the individual particles. Furthermore, he shows that the study of the variations can give precise information on the changes which take place in the individual volume of the particles in suspension during its evolution, at any rate as long as the particles are small enough to permit the use of Lord Rayleigh's formula.

We showed above that this must be the case. When the dimensions increase, the term λ^4 of the formula must be replaced by another λ^m, the exponent m being less than 4 and all the smaller as the particles become larger.[17a]

Now, as we have seen, there is another property of suspensions which is related to the volume of the particles, namely, viscosity. There are three formulas: that of Einstein, that of Arrhenius, and that of Kunitz,[18] which

[17] Boutaric, A., *"Remarques sur l'étude des transformations pouvant se produire dans les milieux troubles,"* *Bull. Soc. Chim.*, 4e série, 49, 389 (1931).

[17a] Boutaric, *Thèse*, Paris, 1918.

[18] See page 32.

link viscosity with the total volume occupied by the particles in the suspension and show that the viscosity depends solely on the total volume of the particles and *not* on the individual volume, that is to say, not on the degree of dispersion of the suspension.

Let us now examine how the coefficient of viscosity η varies during the evolution of a suspension, in the two cases which interest us. (a) If the particles grow by agglomeration without intervention of the solvent, the total volume of the particles does not vary and the viscosity keeps the same value. (b) If the particles grow by swelling in the solvent, the viscosity η, which according to Einstein's formula is a linear function of the total volume φ occupied by the particles contained in one unit of volume of the suspension, is also, if the number of particles is invariable, a linear function of the volume of the individual particles. In the case when viscosity is linked to the volume of particles by the equations of Arrhenius or of Kunitz, the volume φ is still a growing function of viscosity, and consideration of this function: $\varphi = \mathcal{f}\left(\frac{\eta}{\eta_0}\right)$ gives information as to the total volume of the particles.

In all cases, the study of viscosity gives indications which complete those that can be derived from the study of the coefficient of luminous absorption h.

In brief, if in the course of the evolution of a suspension the coefficient of luminous absorption goes on increasing, it must be concluded that the volume of the individual particles has increased, this volume being proportional to h. If the viscosity does *not* vary, it must be concluded that the evolution of the suspension was caused by some coalescence of the particles without intervention of the solvent. If the viscosity *increases*, it means that there has been fixation of the solvent by the particles.

Consequently, the comparison of measurements of viscosity and of the coefficient of luminous absorption (optical density) enables one to study the variations of the number ν of particles in suspension. For, the total volume φ of particles being a function $\mathcal{f}\left(\frac{\eta}{\eta_0}\right)$ of the relative viscosity of the suspension with respect to the solvent, and the coefficient of luminous absorption h being proportional to the volume of the individual particles, V, the quotient:

$$\frac{\mathcal{f}\left(\frac{\eta}{\eta_0}\right)}{h}$$

gives the number of particles.

Table 21 reproduces two series of measurements of the optical density and the viscosity of serum.

In this table we find: (1) The values $\log I_0/I$ of the logarithm of the

Table 21.

Temp. (° C)	$\text{Log}\frac{I_0}{I}$	$\frac{\eta}{\eta_0}$	$f\left(\frac{\eta}{\eta_0}\right)$			$\frac{f\left(\frac{\eta}{\eta_0}\right)}{\text{Log}\frac{I_0}{I}}$		
			E	A	K	E	A	K
50	0.10	1.8	0.7	0.223	0.12	7.5	2.2	1.2
55	0.11	1.8	0.8	0.249	0.13	7.7	2.3	1.1
58	0.11	1.9	0.9	0.283	0.15	8.3	2.5	1.3
60	0.14	2.0	1.0	0.314	0.15	7.8	2.3	1.1
62	0.16	2.3	1.3	0.358	0.18	8.0	2.3	1.1
64	0.21	2.9	1.9	0.464	0.22	9.0	2.2	1.0
66	0.23	4.0	3.0	0.612	0.27	13.5	2.6	1.2

quotient of the initial luminous intensity to the transmitted intensity for a serum heated ten minutes at different temperatures, t, and cooled. (2) The values of the relative viscosity, $\frac{\eta}{\eta_0}$, for serum heated at the same temperatures. (3) The values of the functions $f\left(\frac{\eta}{\eta_0}\right)$ given by the equations of Einstein (E), Arrhenius (A) and Kunitz (K). (4) The quotients:

$$\frac{f\left(\frac{\eta}{\eta_0}\right)}{\log\frac{I_0}{I}}$$

relative to these diverse functions which, as we outlined above (see the original paper for the calculations), are proportional to the number of particles ν.

Table 22 gives analogous results, in which the author combines three series of measurements of optical density and three series of measurements

Table 22.

Temp. (° C)	$\text{Log}\frac{I_0}{I}$	$\frac{\eta}{\eta_0}$	$f\left(\frac{\eta}{\eta_0}\right)$			$\frac{f\left(\frac{\eta}{\eta_0}\right)}{\text{Log}\frac{I_0}{I}}$		
			E	A	K	E	A	K
50	0.105	1.937	0.94	0.287	0.14	8.9	2.7	1.3
55	0.108	1.947	0.95	0.289	0.14	8.8	2.7	1.3
58	0.120	2.007	1.00	0.302	0.15	8.4	2.5	1.2
60	0.140	2.123	1.12	0.327	0.16	8.0	2.3	1.1
62	0.165	2.315	1.31	0.364	0.17	8.0	2.2	1.0
64	0.222	2.649	1.70	0.423	0.20	7.4	1.9	0.9
66	0.238	3.315	2.32	0.520	0.24	9.7	2.2	1.0

of viscosity, the values of log I_o/I and of $\frac{\eta}{\eta_o}$ being the mean of the three determinations.

"Thus, we see that measurements of viscosity and of optical density lead us to admit that the number of particles present in serum does not vary in the course of the transformations produced by heating. (The three last columns give approximately constant figures: the one which corresponds to the formula of Einstein is the least good, but we know that Kunitz showed that it only applied in the case of very dilute solutions,[19] which is far from being the actual case.) *The serum molecules swell by fixation of water and their number does not vary. These results confirm very clearly, therefore, the conclusions of M. Lecomte du Noüy, who started from different theoretical considerations.*"*

It, therefore, seems to be well established that, in spite of its typically colloidal aspect, heated serum contains neither more nor less scattering particles than fresh serum; that there has been neither aggregation nor disaggregation (at any rate for a short time of heating); that the molecules have simply increased in volume; and that their optical anisotropy has been increased.

We can now try to get an idea of the manner in which these water molecules are fixed to the serum molecules so as to explain the preceding phenomena.

We have four principal experimental facts concerning normal serum, namely: (1) The volume of the molecules increases only when the temperature has reached about 55°. (2) The volume of the molecules then increases constantly and almost linearly as a function of the temperature. (3) The factor of depolarization increases in a continuous fashion and attains high values. (4) There is no agglomeration of molecules as a result of heating. The number of scattering particles remains constant.

We have been unable to find in the literature any precision whatever on the mechanism of hydration. The terms employed are usually "hydration," "fixation of water molecules," or "adsorption of water." Marinesco speaks of the thickness of the "perisphere" of water.[20] We did not succeed in grasping clearly the idea of the authors concerning the place where the molecules of water are fixed. This is a detail generally passed over in silence. To our

[19] Kunitz, *J. Gen. Physiol.*, 9, 715 (1926).

* Italics ours.

[20] "In the case of methemoglobin, the mean molecular weight of which is known, we find for the perisphere of water (compressed by dielectric attraction), 70×10^{-8} centimeters, whereas the nucleus of the micelle has a radius equal to 27×10^{-8} centimeters." Marinesco, *Compt. Rend. Acad. Sci.*, 187, 70 (1928).

knowledge it has not been the object of any special study, but we do not pretend to have read everything published on the subject in all languages.

However it seems that, actually, the general tendency is to consider that the water molecules are adsorbed at the periphery of the protein molecule. Let us try to see what would be the consequences if such were the case. As the heating increases a greater number of molecules are adsorbed. The large protein molecule is finally surrounded by a layer of water the thickness of which goes on increasing until, as our experiments and calculations showed, it can attain a value equal to about one-half the "mean diameter" of the normal serum molecule, *i.e.*, about the thickness of 20 molecules of water (H_2O, monohydrol). When this thickness is reached, the total volume of the molecule, including its water envelope, is multiplied by 9 and there is contact between the water which is fixed on all the molecules: the serum is coagulated. Now, according to this theory, it is not the molecules themselves which are in contact, but the water of adsorption.

What is the difference then between the serum in a liquid state, where the molecules are at the same distance from one another, and the gel, in which nothing is changed except that the water is "adsorbed"? What is the significance of this mysterious force of adsorption which can make itself felt through a thickness of about 40 layers of water molecules (twenty on each molecule)? The usual answer is: pure electrostatic action. A gel would thus be constituted of water immobilized by forces and would not be a structure. It would then be difficult to understand why the optical anisotropy of the molecule, revealed by the measurements of the factor of depolarization, should increase constantly and more and more rapidly as coagulation progresses. It would even be difficult to understand why the quantity of scattered light (proportional to the square of the volume of the particles) should increase as a function of the temperature, unless we admit that the optical properties of the fixed water differ profoundly from those of free water. What is more, it would be hard to understand the nature of the differences between the water which surrounds the protein, which is no longer liquid, and the liquid water in which it floats:[21] what is admissible for a monomolecular or a bimolecular adsorbed layer is inacceptable when the layer is 20 Angstroms thick. Finally, why should a rise of 1° in the temperature determine the fixation of a new layer of water on the others, and why does this adsorption take place only above a certain temperature (56°) and not below?

It is evident that this hypothesis is as difficult to accept as that of coagu-

[21] Electrostatic attraction, which has been called in to explain these facts, is not satisfactory. It is not clear how this attraction could increase regularly by an approximately equal quantity for each degree of temperature. It is not an explanation, but a word.

lation by aggregation of molecules which, as we have seen, is in absolute contradiction to the experimental observations.

But another hypothesis can be put forward which, though seemingly heterodox, is in reality quite classical and has the advantage of explaining all the preceding experimental facts. This hypothesis consists simply in admitting that the water is adsorbed inside the protein molecule, between the ramified chains which constitute its complex structure.

To penetrate into the interior of this structure, which is protected by a field of forces between the external groups, the small molecules of water must possess a certain ballistic energy. A stone thrown by hand does not penetrate an obstacle which is easily pierced by a bullet. This ballistic energy is supplied by the temperature, and this explains the hitherto mysterious fact that below 54° the serum is practically unalterable, no matter how long the duration of the heating. Below a certain temperature the kinetic energy of the molecules of water is not great enough to enable them to overcome the peripheric forces of the protein molecule. But as soon as the energy has exceeded a certain value, which is precisely determined by the magnitude of the forces of attraction between the water molecules, the latter force an entrance and infiltrate themselves between the atomic ramifications. If the temperature does not increase beyond this value, the kinetic energy of the molecules, which is proportional to the temperature, remains constant. The molecules can penetrate only to a certain depth, and for low and medium temperatures (55° to 58°) a state of equilibrium corresponding to a kind of saturation is probably reached when the heating is maintained long enough. In other words, the increase in volume of the particles is not proportional to the time of heating, but tends toward a constant value. The curves which express this phenomenon should have an appearance of convexity toward the axis of ordinates (volumes) and finally take on an asymptotic aspect if the heating is prolonged. On the other hand, for high temperatures, beyond 60°, the tendency of the curve to settle at a constant value should decrease, as a result of the deformation which takes place and of the weakening of the peripheric molecular forces, and should entirely disappear at the higher temperatures. This is exactly what the experiments show (Fig. 27).

If, on the other hand, the time of heating is the same and the temperature is raised, the kinetic energy of the molecules of water increases and they penetrate more deeply into the interior of the structure and in greater numbers. Their number should be a direct function of the force of penetration, and the curve representing the increase in the volume of the protein molecules as a function of temperature should be practically a straight line. This is also what the experiments show (Figs. 38 to 42). As the number of water molecules which strike the protein molecule increases proportionally to the

temperature, the increase in the volume of the latter is a linear function of the temperature, which is a direct consequence of the preceding observation.

This hypothesis, therefore, supplies an explanation for the stability of the serum up to about 55°, and its subsequent behavior, up to complete coagulation. However, it alone cannot explain the irreversibility of these phenomena. Indeed, as temperature is a statistical phenomenon, there are molecules of water which have a sufficient kinetic energy even at temperatures below 55° to enable them to penetrate a serum molecule. If the phenomena were reversible one could understand how an equilibrium could be reached. But above 55°, or thereabouts, the molecules of water are irreversibly fixed. Besides, the phenomena which we measure are also statistical and the time of heating also plays a part, even though a slight one. Four hours of heating at 50° determines a slight increase in the rotatory power, for instance; it is therefore necessary to admit that a temperature of approximately 55° determines a slight chemical change in the structure of the protein such that the molecules of water, when they have penetrated into the interior, are definitively, chemically fixed, which would explain the threshold of 55° and the irreversibility. This hypothesis is in accord with the fact that the curves which express the viscosity as a function of the temperature show that the phenomenon is reversible as long as the heating has not been carried beyond 55°.

In brief, as everything takes place as if the molecules of serum were hydrated from the interior much more than from the exterior, and swelled like a cube of gelatin, all the transformations that we described in the optical properties become comprehensible: especially the continuous increase of the rotatory power and of the factor of depolarization. Indeed, as we have shown,[22] the rate of increase of the rotatory power as a function of the temperature (increase of the angle of rotation per minute for heatings of five minutes) is very nearly a linear function of the temperature. This can be very easily explained if it is admitted that the bombardment and penetration of the water molecules progressively separate the ramifications and chains of the protein, thus modifying the previously existing symmetry. This very regular modification manifests itself by the continuous increase of the rotatory power, proportionally to the temperature. As the swelling progresses, the relative place of the chemical groups varies in space, as if they were fixed on a spiral, the length and diameter of which would grow simultaneously without necessarily modifying the length of the principal chain or chains. The spirals of an elastic coil-spring give an idea of these deformations. It is, moreover, possible that the fixation of water on certain internal groups also contributes to modify the rotatory power.

It would be impossible to explain the continuity of this phenomenon

[22] See pages 43 and 44.

of rotation of the polarization plane or even the phenomenon itself, if the coagulation were due to a micellar aggregation. The only hypothesis which enables one to interpret the observed facts is that of a molecular dispersion in the serum, that is to say, the concept of a true solution which is maintained until coagulation.

The rapid increase of the optical anisotropy, revealed by the considerable increase of the value of the depolarization factor, is thus very simply explained by the relative displacement in space of the atomic groups and the penetration of the water molecules.

From a biological point of view it is clear that the progressive separation of the groups could modify totally the chemical properties of serum. From this we must conclude that the complementary power very probably depends on the superficial groups of certain molecules which are easily displaced and perhaps dissociated by violent impacts, whereas the sensitizer, a specific property of the molecule, depends on smaller and more stable chemical groups and therefore is affected only when the water molecules can penetrate deeply enough to alter the molecular architecture of these groups definitively or simply to destroy the links which unite them to the whole.

The curves expressing the values of ρ clearly show that it is between 62° and 66° that the deepest modifications of symmetry probably take place.

Polarimetry is a more sensitive method than the one described in this chapter and shows that for heatings of the order of 60 minutes the phenomenon begins around 50°.[23] However, by virtue of this very sensitivity it is possible that these changes of symmetry at a low temperature are due to local atomic alterations and not to a penetration of the water molecules. In other words, they would reveal the phenomena of peripheric displacement which influence the volume of the molecule[24] only to a slight degree.

We had thought that it should be possible to put in evidence a change in the structure of the serum molecule (increase of a diameter with respect to the others) by measurements of the magnetic birefringence. The experiments would have been an argument in favor of the fact that the increase of the depolarization factor was due to a change in the shape of the molecule. With this in view, we conducted a series of experiments with the large electromagnet of the Academy of Sciences at Bellevue which Professor Cotton kindly put at our disposal and in which he himself participated.

[23] See page 43.

[24] We have intentionally avoided speaking of the action of electrolytes. Indeed, we are studying a relatively stable complex, serum, possessing particular properties which are about constant on condition of not being manhandled. This eliminates a whole series of experiments the immediate result of which would be the destruction of the serum as a physico-chemical system. Nevertheless, we decided to try to get some idea of the behavior and role of electrolytes with the help of physical methods; this work will be dealt with in another chapter.

Three samples of serum were placed in a magnetic field of the order of 50,000 gauss (intensity of the current, 400 amperes). One was unheated and the others heated at 58° and 64° for ten minutes. No trace of birefringence was observed. This seems to demonstrate that the geometrical symmetry of the molecule is not measurably modified by heating. The considerable increase in the value of ρ, therefore, can not be solely due to the increase in volume, as we showed further back, nor in all probability to a change in shape. It can therefore be determined only by an important modification of the optical anisotropy of the molecules, which is a necessary consequence of the relative displacements of the atomic groups due to an expansion by hydration.

We can deduce another conclusion from the preceding pages and the experiments described in this chapter. If we refer to the works of Mie, we see that the quantity of depolarized light attains 10 per cent when the particles are of the order of 130 mμ. Now, we regularly observed values of ρ varying from 50 to 70 per cent and more; nevertheless, the particles probably do not attain lengths of the order of 100×10^{-8} centimeter, or 10 mμ. We are still more than 10 times below the values given by Mie. Consequently, in this particular case there is another argument in favor of the change in structural symmetry and in the optical anisotropy of the protein molecules as a result of hydration, for if the increase were produced solely on the periphery it would be symmetrical.

We are therefore led to admit that the effect of an elevation of temperature manifests itself principally by a penetration, a bombardment of the water molecules toward the interior of the serum molecules, the consequence of which is the increase in volume of the latter as a result of the unfolding of the internal chains which normally exist, probably in the shape of spirals, the carbon valences being separated from one another by an angle of 109°. This hypothesis is not in contradiction with the theory of Debye-Hückel (perisphere of adsorbed water), for this is valid only for molecules which can be assimilated to spheres and which carry a punctual electric charge. Neither of these conditions can be applied to the protein molecules, which are asymmetrical, anisotropic and composed of a large number of amino acids linked together.

This hypothesis of coagulation by intramolecular hydration can be subjected to a crucial test which we had conceived before it was made possible by the remarkable inventions of Mr. James Basset. This test is the following. We stated above that the threshold of 55° was conditioned by perimolecular forces and by the mutual attraction or force of cohesion between the water molecules (11,000 atmospheres).[24a] Now, Prof. Macheboeuf and

[24a] See Willows and Hatschek, "Surface Tension and Surface Energy," p. 13, London, Churchill, (1923).

Dr. Sandor used the ultra-pressure pumps of Basset, which can attain the really formidable pressure of 24,000 atmospheres,[25] and submitted the serum to pressures varying between 1,000 and 13,000 atmospheres. They found, to their surprise, that the serum coagulated around 11,000 atmospheres.[26] It seems at first sight that our hypothesis alone could explain this curious phenomenon. It is evident that the mechanism of hydration through thermic bombardment is not the same as that of hydration through pressure. In the first case it is the kinetic energy of the water molecules which intervenes, while in the second—the mean kinetic energy being constant—it is solely the quantity of movement. Nevertheless, the result is the same when one reaches ultra-pressures which are of the order of magnitude of the cohesion of water molecules (11,000 atmospheres).

Note on the Definitions and Symbols Employed in this Chapter

Depolarized Light. There is an *apparent* contradiction between the terms employed which can be easily explained. The total scattered flux F_τ includes:

(1) A flux F_n of natural light.

(2) A flux F_v of polarized light with vertical vibrations.

Thus $F_\tau = F_n + F_v$. After passing through the nicol which stops the vertical vibrations, this flux will become f_H, polarized in the horizontal plane.

Consider $f_H = \frac{F_n}{2}$, (the losses by reflection due to the nicol being neglected).

After passing through the nicol, which stops the horizontal vibrations, the flux will become f_v, polarized in the vertical plane: $f_v = \frac{F_n}{2} + F_v = f_n + F_v$.

The flux F_n of depolarized light, therefore, has a value which is double that of the flux f_H transmitted by the nicol transparent to horizontal vibrations.

When we define the factor of depolarization by the ratio: I_h/I_v, we are really speaking of the ratio f_H/f_v.

Moreover, in all the measurements made with the V.B.Y. photometer the notions which intervene are those of a luminous *flux*, of *luminance* and of *illuminance*, but not those of *intensity* in the strict sense of the word. If we used the symbols I_o and I it was because this book is intended mostly for biologists and biochemists and that it seemed simpler not to change the symbols to which they are accustomed. When the reader is warned of this fact there can be no confusion.

[25] The pressure at the interior of a 16-inch gun at the moment of the explosion is of the order of 4,500 atmospheres.

[26] Basset, Macheboeuf, and Sandor, *Compt. Rend. Acad. Sci.*, 197, 796 (1933).

Similarly, on page 72 we refer to the absolute value of ratio I_o/I. It would evidently be more correct to speak of the absolute value of the ratio:

$$\frac{b\ (\textit{luminance}\ \text{of the diffusing surface})}{E\ (\textit{illuminance},\ \text{expressed in Lux})} = B\ \text{in candles by square meter}$$

(See Fig. 30). For, by substituting a perfectly diffusing surface (coating of magnesium oxide, for instance) for X, this perfectly diffusing surface takes, under an illuminance E, a luminance B such that $B = \frac{E}{\pi}$. As the photometer can measure $\frac{b}{B}$ it is possible to deduce $\frac{b}{E}$.

This terminology corresponds to the recommendations of the Colorimetry Committee of the Optical Society of America (p. 246 of the Journal of the Society, May 1944). However, Professor Arthur C. Hardy, of the Massachusetts Institute of Technology, who favors these terms and to whom we are indebted for this information, points out that, in the terminology of the Illuminating Engineering Society the above equation would read:

$$\frac{b\ (\textit{brightness}\ \text{of the diffusing surface}\ X)}{E\ (\textit{illumination},\ \text{expressed in Lux})} = B$$

Chapter 7

Ionic Equilibria of Serum as a Function of Temperature. I. Sedimentation

So far, we have not taken into account any of the correlative ionic phenomena which probably play an important part in the facts we have studied. We left them aside purposely so as to simplify the problems arbitrarily, and to get a comprehensive view and a general idea of the evolution of the physical and physico-chemical phenomena subsequent to heating. We shall now describe as simply as possible the preliminary experimental results obtained by studying ionic phenomena.

Precipitation of Globulins by Addition of Water.[1]

Unheated Serum. It has been known for a long time that the stability of certain proteic elements of serum (globulins) depended, among other factors, on the concentration of salts. When serum is diluted with distilled water, a cloudiness generally appears, which is followed by the precipitation of part of the globulins. Nothing of the kind is observed when the dilution is made with a 0.9 per cent isotonic physiological solution. The decrease in the salt concentration, therefore, brings about a decrease in the solubility of the globulins, if we admit that they exist in serum in a free state, or a separation from the serum molecule, if we admit that they are normally combined.

Two possible mechanisms of the phenomenon can, therefore, be considered: either there is a true insolubilization, which can be caused by a change in the ionization of the globulin chlorides and other globulin salts existing in a free state in the solution; or else the complex serum molecule simply separates in two: soluble albumin on the one side and insoluble globulin on the other. The splitting of these two molecules would, in itself, entail precipitation of the molecule which, though *normally insoluble*, derived an apparent solubility from the fact that it was combined—no matter how—with another substance, (albumin) molecularly dispersed. This question will be studied later. Actually we simply propose to give the experimental results obtained by studying quantitatively the phenomenon of precipitation after the addition of distilled water to normal, unheated and heated serum.

[1] Lecomte du Noüy, *Ann. Inst. Pasteur*, 48, 187 (1932).

Table 23. Light Scattered by a Normal Horse Serum After Addition of Distilled Water

No. cc water added to 1 cc serum	Relative concentration in salts (%)	Readings: Log $\frac{I_0}{I}$	
		After 1 hour	After 4 hours
Pure serum	1	2.18	2.23
+ 1	0.50	2.12	2.14
2	0.33	2.06	2.08
3	0.25	1.54	1.50
4	0.20	1.30	1.30
5	0.168	1.17	1.21
6	0.143	1.15	1.16
7	0.125	1.12	1.14
8	0.115	1.12	1.14
9	0.100	1.13	1.15
10	0.091	1.15	1.17
11	0.083	1.17	1.19

The method employed has the advantage of being very rapid, and of being at least as accurate as the weighing method, which requires a great many manipulations and thus introduces a large number of causes of error.

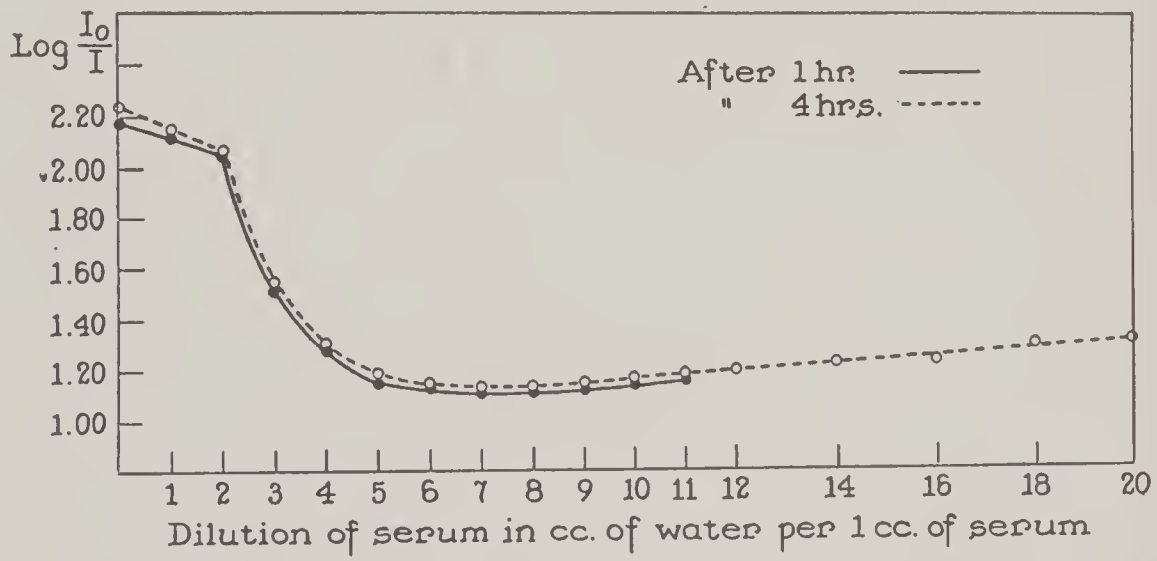

Fig. 43. Light scattered laterally by a normal horse serum after addition of varying quantities of distilled water. The figures representing the values of log $\frac{I_0}{I}$ *decrease* as the scattered light *increases*, that is, as the solution becomes more and more cloudy.

The quantity of light scattered at right angles by the solution was determined with the instrument already described.[2] To monochromatize the light we used a blue Wratten filter No. 45H, which allows the passage of a dominant wave length of 4,800 Angstroms. The figures obtained express the

[2] See page 67.

value of log I_o/I, *i.e.*, the ratio of the intensity of incident light to that of scattered light. It is known that this value is proportional to the number of scattered particles (when the latter are very small) and to the square of their volume (assuming that their shape does not vary much from that of a

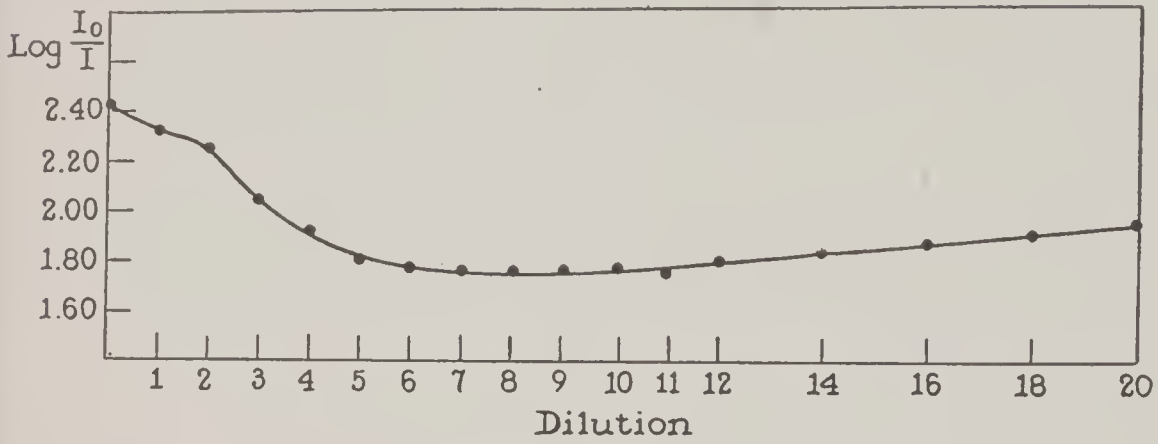

Fig. 44. Same experiment as that shown in Fig. 43 but with a different serum. The phenomenon is less marked.

sphere). Table 23 expresses the results of a series of measurements and gives the general aspect of the phenomenon (Figs. 43 and 44).

The measurements were made at intervals of one and four hours after the preparation of the solutions, which were stirred so as to insure homogeneity. From 0 (undiluted serum) to 200 per cent (1 cc of serum+2 cc of water), the quantity of scattered light increases very little and remains

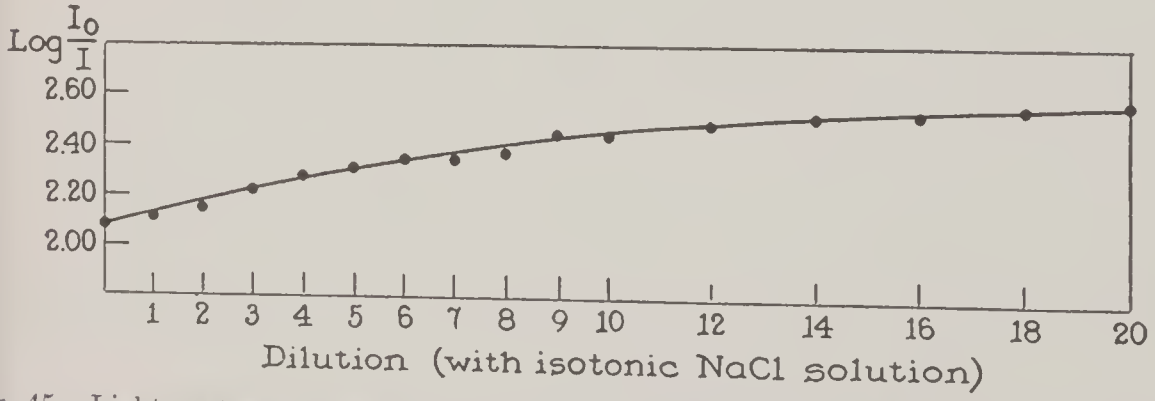

Fig. 45. Light scattered laterally by a normal horse serum after addition of varying quantities of physiological solution. The scattered light decreases with the dilution.

approximately proportional to the dilution. Beyond 200 per cent there is a sudden increase clearly shown by the curves which thereafter assume a logarithmic aspect. Fig. 45 shows, by comparison, the aspect of the curve when the serum is diluted with the physiological solution instead of distilled

water. It can be seen that log I_o/I increases, that is to say that I, (scattered light) decreases.

Around 500 per cent (5 cc of water+1 cc of serum), the curve reaches a minimum. This low value is usually maintained until around 900 per cent, when the scattered light begins to decrease. (The figures in the table express log I_o/I and are inversely proportional to the intensity of the scattered light, which explains the fact that they first decrease and then increase). The addition of 5 cc of water to 1 cc of serum can increase the scattered light in the proportion of 1 to 13.

It is evident that the reason why the phenomenon is so clear is the high concentration of insoluble globulins in horse serum. Following are the mean quantities of globulins in different sera, according to physiological and biochemical textbooks:

Horse	4.3 per cent
Ox	3.5 " "
Sheep	2.3 " "
Pig	1.8 " "
Rabbit	2.0 " "

The curve obtained with ox serum is almost analogous to that with horse serum, but the break corresponding to a dilution to ⅓ of the initial salts, which characterizes the horse serum curve, is not observed. The curves given by the sera of other animals which are poor in globulins (about half the amount contained in horse serum) are totally different. They are con-

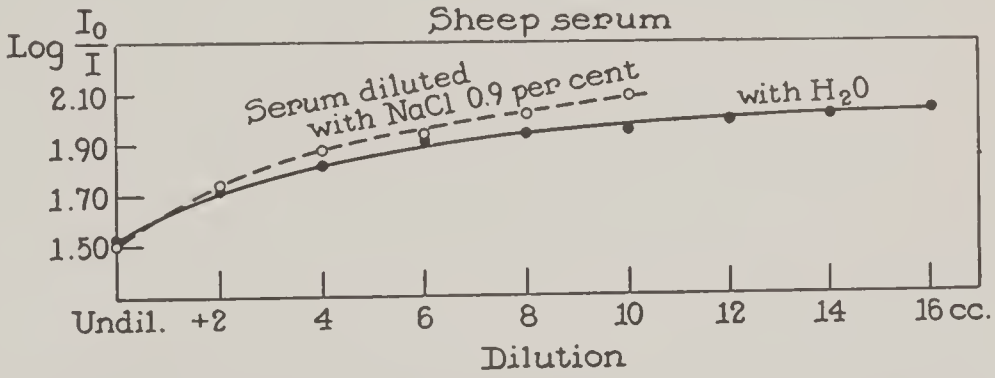

Fig. 46. Experiment similar to those of Figs. 43, 44, 45, but with sheep's serum having a slight content in globulins.

vex instead of concave, and this convexity is very similar to that obtained by diluting serum with isotonic solution at 0.9 per cent. Fig. 46 gives the results obtained with sheep serum.

It may be noted that the more frequently the horse has been bled the clearer the phenomenon becomes. Everything takes place, in brief, as if repeated bleedings resulted in an increase of the insoluble portions of the serum.

Action of Temperature

When horse serum has been heated for ten minutes at different temperatures, the aspect of the curves expressing the precipitation of globulins is modified. Figs. 47 and 48 show that the quantity of scattered light, that is,

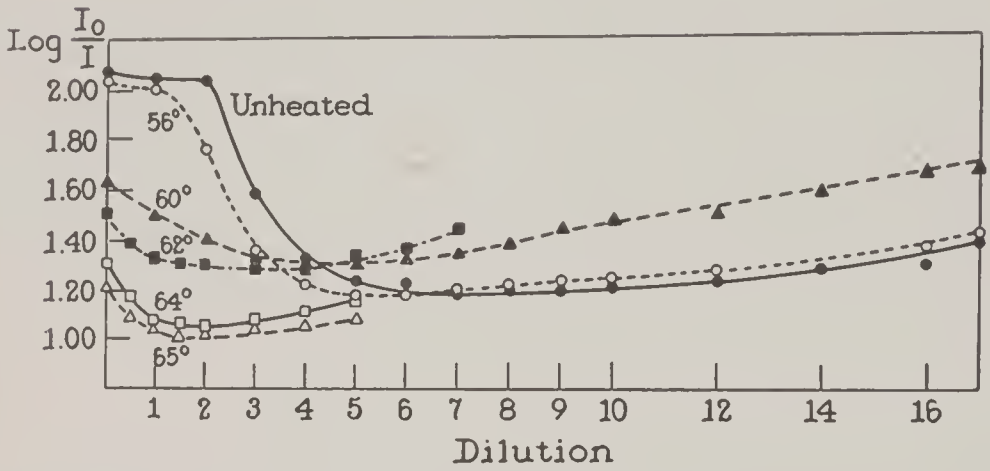

Fig. 47. Light scattered by normal horse serum, unheated (solid curve) and heated, as a function of the dilution with distilled water. The abscissae express the number of cubic centimeters of water added to 1 cc. of serum. Time of heating 10 minutes.

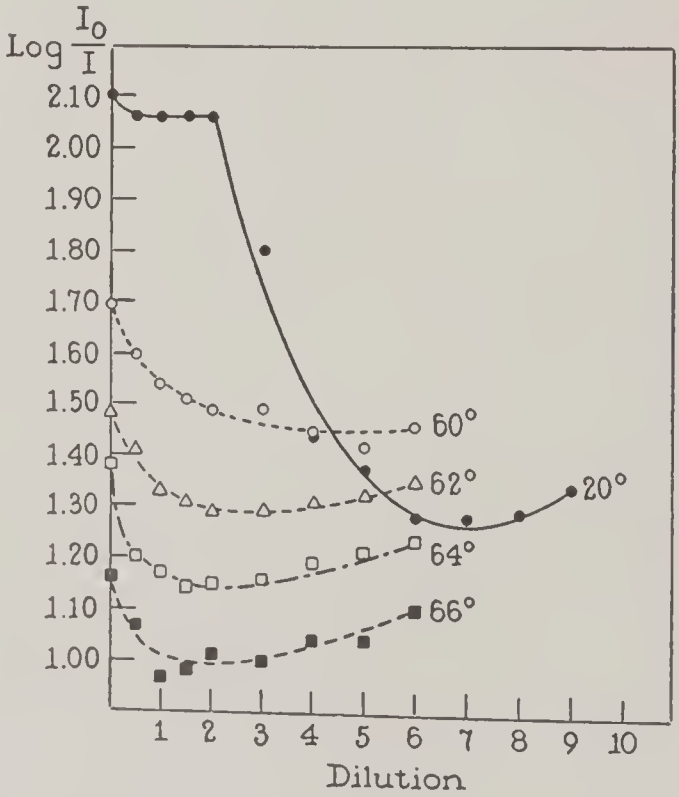

Fig. 48. Same experiment as in Fig. 47. The scale of ordinates has been increased so as to make the curves clearer. 10 minutes heating.

the number or the volume (or both) of light-scattering particles, is increased. Moreover, the refractive index of the particles varies as a result of the instability brought about in the solution, followed by insolubility or separation

of the elements. Therefore, the factor between parentheses $\left(\frac{n_1^2 - n^2}{n_1^2 + 2n^2}\right)^2$ which could be neglected in the preceding chapter, becomes important and it is impossible to calculate the values of $\frac{dV_1}{dV}$.

Each temperature corresponds to an optimum dilution for which the quantity of light laterally scattered at 90° is maximum. This maximum may be due to the increase in volume, to the increase in the number (as a result of disaggregation), and to the increase of the refractive index of the insoluble, light-scattering particles, as we said above. The curves show clearly that the higher the temperature, the less the serum must be diluted

Table 24. Light Scattered by a Horse Serum Heated for Ten Minutes after Addition of Distilled Water.

No. of cc water added to 1 cc of serum	Readings: Log $\frac{I_0}{I}$			
	Unheated Serum	Heated at 56°	Heated at 60°	Heated at 64°
0	1.98	1.96	1.72	1.19
+0.5	1.98	1.96	1.62	1.09
1	1.99	1.92	1.59	1.05
1.5	1.98	1.84	1.54	1.00
2	1.95	1.68	1.53	1.03
3	1.63	1.56	1.39	1.05
4	1.33	1.47	1.32	1.04
5	1.25	1.40	1.31	1.07

in order to obtain the maximum of precipitation. For the particular serum employed in the experiment represented in Fig. 47, this maximum is obtained at a dilution of 700 per cent in the unheated serum and of 150 per cent in the serum heated at 65°. Either the insolubility of the globulins increases, or else the complex albumin-globulin becomes more fragile. The shift of the maximum begins around 50°. (We remind the reader that this maximum corresponds to a minimum in the figures, as the measurements express the value of $\log I_o/I$). But Fig. 49 shows that, in the serum studied, the general aspect of the curves is modified profoundly only above 57°. Fig. 50 gives the general aspect of the phenomenon of shift, *i.e.*, for each temperature, the dilution which corresponds to the maximum of scattered light, for a given horse serum heated before dilution.

Redissolution of the Precipitate

The globulin precipitate is generally completely redissolved by adding NaCl. When normal horse serum has been diluted by addition of five times its volume of distilled water, it suffices to bring the salt concentration back

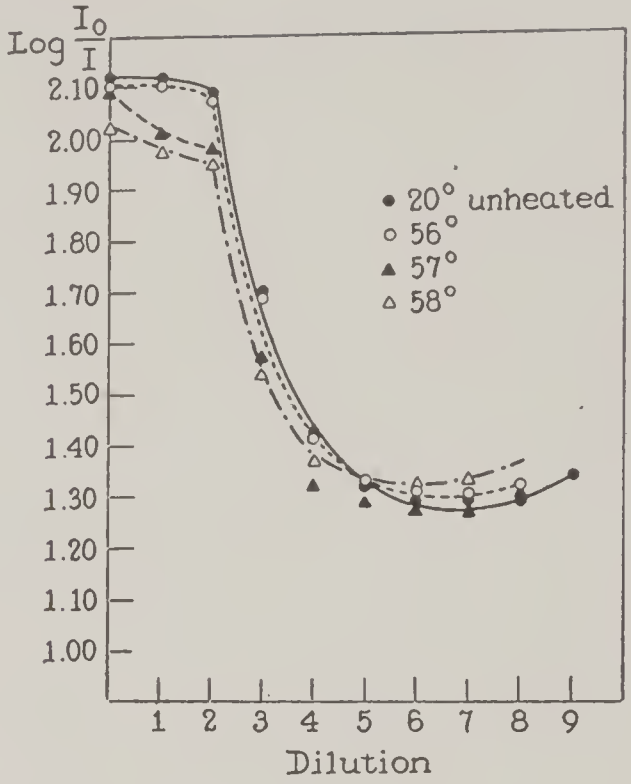

Fig. 49. Same experiment as Fig. 48, but for temperatures not exceeding 58° C. 10 minutes heating. It may be seen that for this serum, the same as in Fig. 48, the action of the heat is slight, up to 56° C. and does not become important before 58° C. *See Fig. 48*

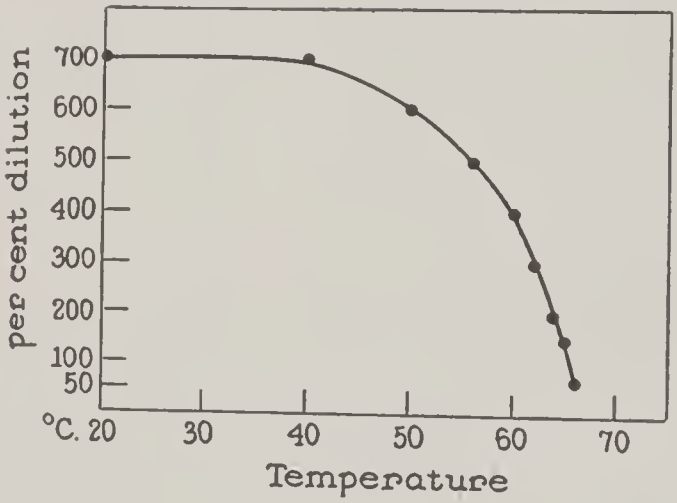

Fig. 50. Curve expressing, as a function of temperature, the shift of the minima of curves similar to those of Figs. 47, 48, 49. These figures show that the curves corresponding to a determined temperature, fall and rise again. The minimum point (which corresponds to a *maximum* of scattered light) changes for each curve and corresponds to a given dilution. The present curve expresses these changes, always in the same direction, when the temperature rises, for a given horse serum. It shows that the scattered light is maximum for a dilution of 400 per cent when the serum has been heated to 60° C., for 10 minutes, whereas this maximum takes place at 700 per cent when the serum has been heated to 40° C.

to between 30 and 40 per cent of the initial value with NaCl, to cause almost complete dissolution. A more or less horizontal plateau is observed (Fig. 51) and dissolution is approximately total at 100 per cent. (The readings on the photometer reach their original value.)

The intermediary quantities of NaCl are less efficacious when the diluted serum has been slightly acidified (0.1 cc to 0.3 cc of a solution of 0.1*N*HCl) so as to determine a more abundant precipitation; but when 100 per cent is

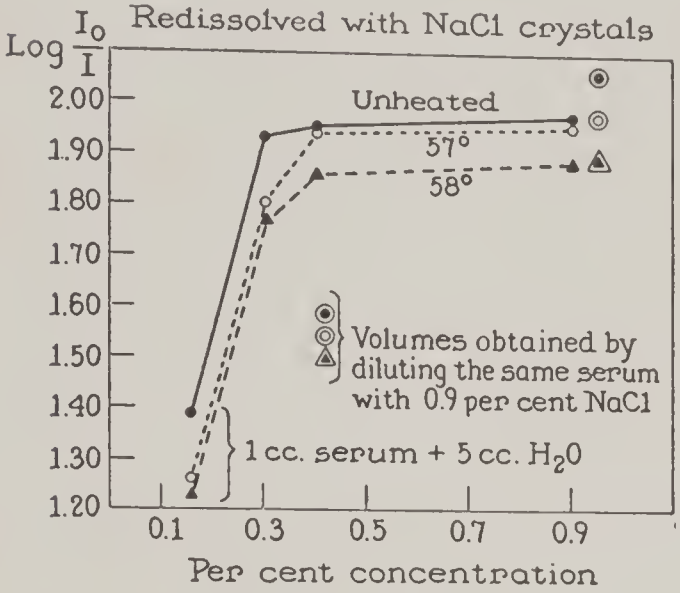

Fig. 51. Redissolution of the globulin precipitate by addition of NACl crystals. It may be seen that the redissolution is almost complete as soon as the salt concentration reaches around 0.4 per cent.

reached the dissolution is likewise practically complete. When a 1 per cent solution of NaCl is used, the proteins are diluted and the salt concentration is increased. This is expressed in the curves of Fig. 52, which show that re-

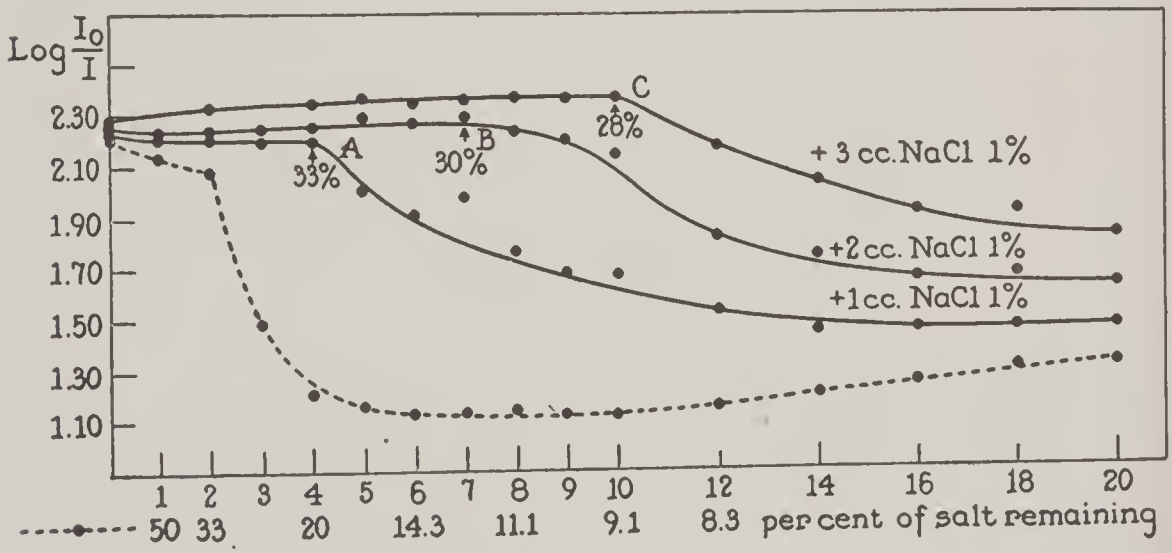

Fig. 52. Redissolution of the globulin precipitate by addition of NACl crystals. It may be seen that the redissolution is almost complete and hardly increases when the concentration is increased.

dissolution is always almost complete when the concentration is brought back to 30 per cent. In this experiment the redissolution was produced with different quantities of a 1 per cent NaCl solution (1, 2, and 3 cc). The

points of inflection A, B, C, of the curves (corresponding to the limit of practically total redissolution) are found at concentrations of 33, 30 and 28 per cent of the normal concentration of salts. This value was, therefore, critical for this serum (normal horse serum), as was shown in Figs. 43 and 44.

Rate of Sedimentation of Globulins

The globulin precipitates formed by the addition of water show a distinct difference in their aspect according to the temperature at which the serum has been previously heated. In order to obtain quantitative measurements, instead of a necessarily imprecise and subjective description, we measured the rate of sedimentation of the precipitate by using the same technique as

Table 25. Experiment No. 16.

Time	Readings: Log $\frac{I_0}{I}$			
	Unheated	Heated ten minutes at:		
		56°	57°	58°
0 min.	1.33	1.31	1.26	1.25
5 min.	1.33	1.32	1.25	1.25
10 min.	1.34	1.32	1.25	1.24
15 min.	1.34	1.33	1.25	1.24
20 min.	1.34	1.32	1.25	1.24
25 min.	1.35	1.32	1.24	1.24
30 min.	1.66	1.33	1.24	1.24
35 min.	1.73	1.28	1.23	1.25
40 min.	1.86	1.32	1.14	1.24
45 min.	1.91	1.72	1.13	1.24
50 min.	1.95	1.82	1.55	1.25
60 min.	1.97	1.93	1.66	1.25
75 min.	2.04	2.03	1.77	1.25
90 min.	2.10	2.03	1.80	1.26
105 min.	2.15	2.03	1.85	1.26
2 hours	2.21	2.03		1.27
3 hours				1.29
5 hours			2.05	
7 hours			2.15	
18 hours	2.42	2.31		1.45

before, *i.e.*, by measuring the relative intensity of the scattered light at right angles by the solution. The readings (again corresponding to the values of log I_0/I) were made every five minutes over a period of three hours or more. Table 25 gives the results of a series of measurements made on fresh normal horse serum, diluted to five times its volume with distilled water (very slightly acid).

This dilution, as we showed in our preceding experiments, corresponds approximately to a minimum in the curve representing log I_0/I (Table 25 and Figs. 53 and 54, Exp. Nos. 26 and 38).

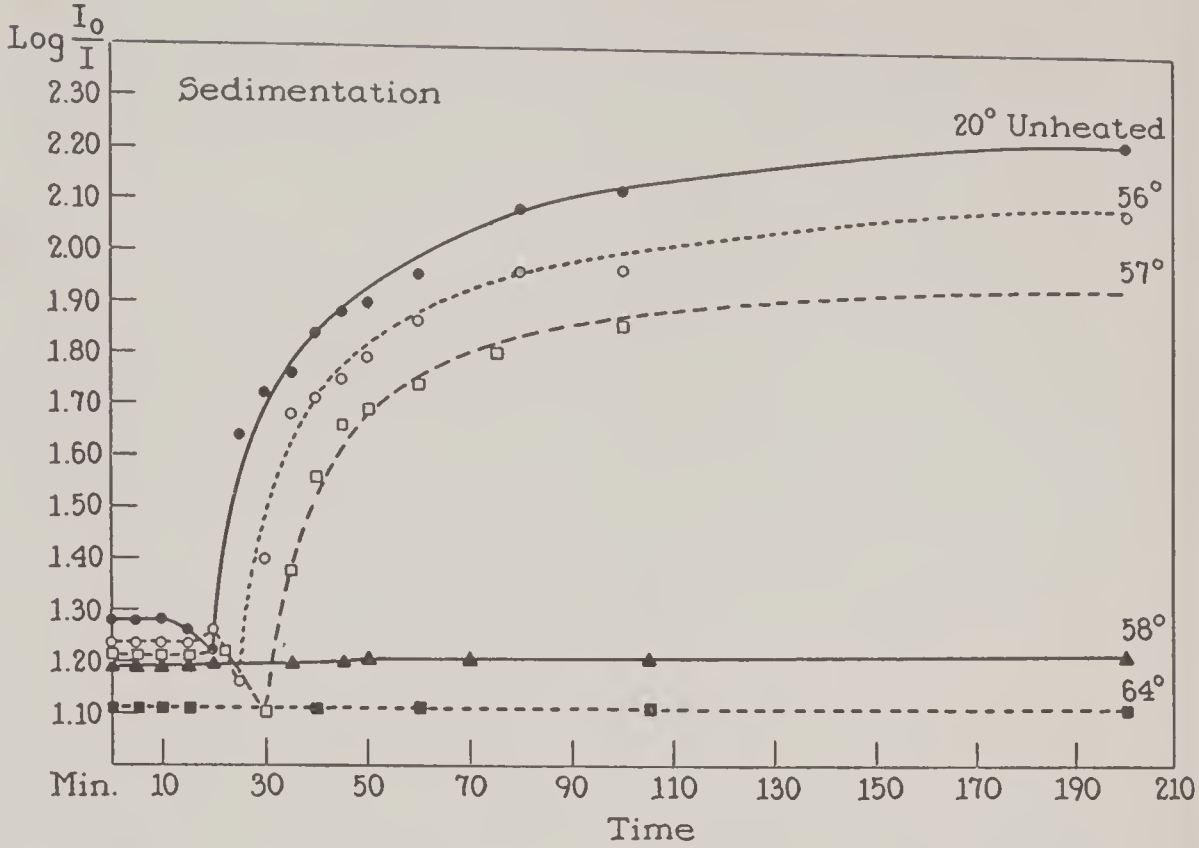

Fig. 53. Sedimentation of globulins. The curves express the progressive clearing of the serum solution. They show that serum heated to 57° C. for 10 minutes, clears, that is to say that the globulins settle, whereas at, and above, 58° C., the solution has a milky appearance and is stable.

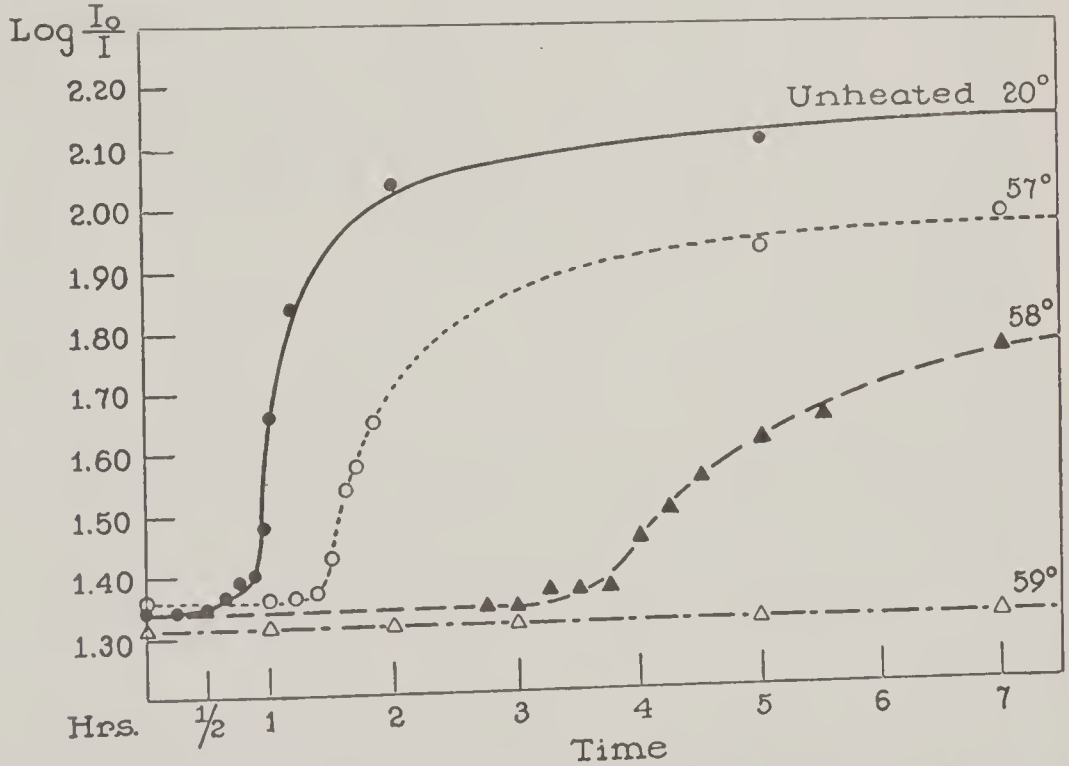

Fig. 54. Same experiment as in Fig. 53 (different serum). With this particular serum the phenomenon was greatly delayed.

It will be noted that the sera heated at 56° and 57° (then cooled) show a slight decrease in log I_o/I which corresponds to a momentary *increase* in the scattered light at the end of 35 minutes in the first case, and 40 minutes in the second. This increase is followed by a sudden *decrease* of the scattered light, which goes on following a regular curve, practically parallel to that of unheated serum. This phenomenon simply expresses the progressive clearing of the solution as the precipitated globulins sediment at the bottom of the cell. But the important and very significant fact is that nothing of the kind happens when the serum has been heated at 58°. In other words, in the latter serum (58°) *there is no sedimentation*: the globulin suspension is stable. The beginning of an obvious clearing takes place only after 18 or even 20 hours. For fresh serum, unheated or heated below 58°, the rate of

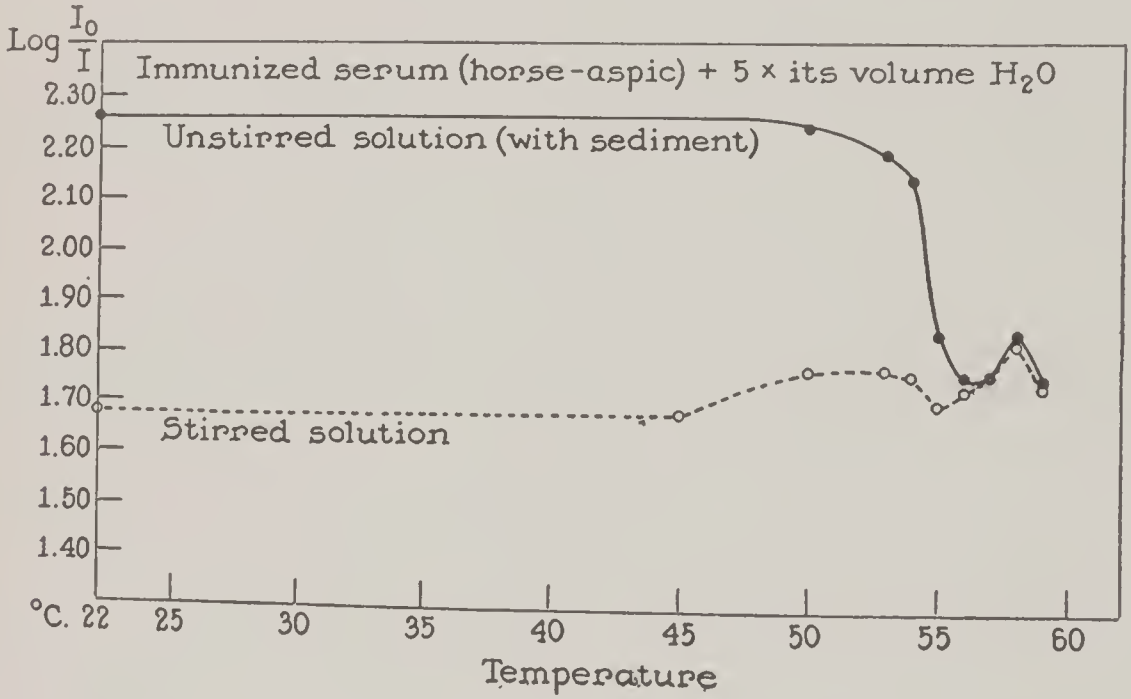

Fig. 55. Light scattered by a solution of serum diluted to 500 per cent, as a function of the temperature to which the serum had been heated beforehand for 10 minutes. The upper curve (solid dots) expresses the values obtained when the solution has not been stirred and the precipitate has settled at the bottom of the cell. The lower curve (white disks) expresses, on the contrary, the values obtained with the same solutions when stirred so that the precipitate is again in suspension. The curves join at 57° C. The stirring no longer plays a part and the suspension is stable.

sedimentation is considerable, as at the maximum (between 25 and 30 minutes for the unheated serum, between 40 and 45 for serum heated at 56° and between 45 and 50 for serum heated at 57°) the number of scattering particles is decreased by more than half, in a given volume, in five minutes. (It must not be forgotten that the figures in the table represent differences between logarithms). However, we cannot interpret these data so as to under-

stand completely the mechanism of the phenomenon, for Rayleigh's formula applies only to particles which are small with respect to the wave length of light, and although we do not yet know the order of magnitude of these particles we know that they must be much larger.

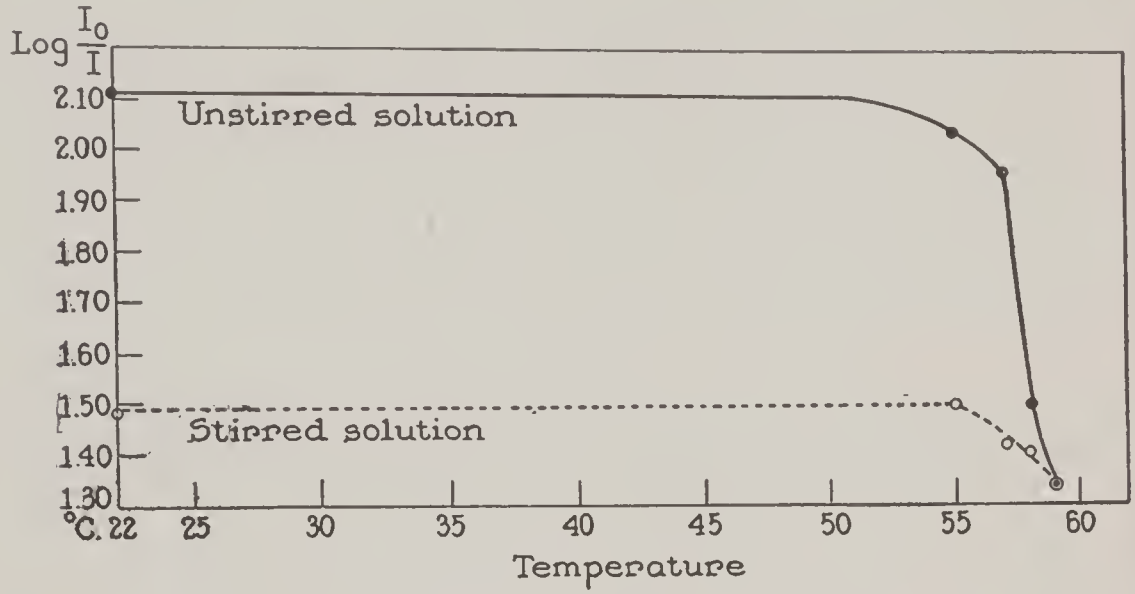

Fig. 56. Same experiment as Fig. 55. Different serum.

A new phenomenon can, therefore, be added to the four already pointed out (viscosity, rotatory power, scattered light, factor of depolarization) which characterize the important physico-chemical modifications undergone

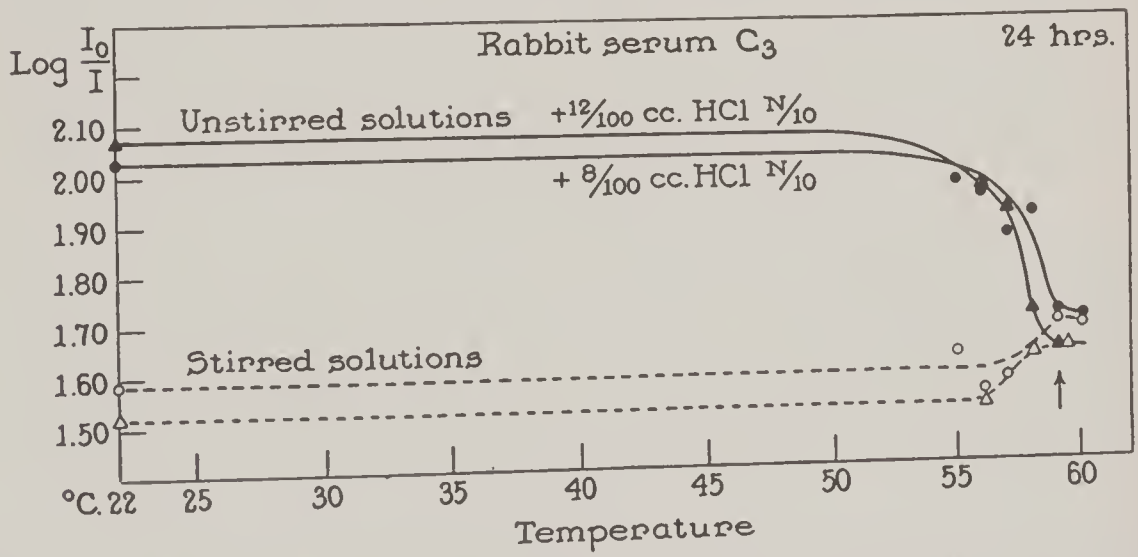

Fig. 57. Same experiment as those of Figs. 55 and 56, but with rabbit serum and after slight quantities of acid have been added.

by a serum heated above 57°. But the last one affords an interesting precision, inasmuch as it shows that, as we already suspected, it is the globulins which are most profoundly affected between 57° and 58°. This temperature,

however is not absolute: certain horses show the same reaction between 58° and 59°, others between 56° and 57°. But a difference of one degree is *always sufficient* to bring forth a strikingly clear difference between the curves.

This phenomenon which, in its broad outlines, was observed regularly, does not always take place at the end of the same period of time. In certain cases it appears at the end of four hours. In one case, 18 hours passed before sedimentation set in. The phenomenon is generally clearer when the solution is acidified by 0.1 cc of 0.1*N* HCl. It is evident that the pH value plays an important part; but the acidification must be slight, for we know that a small increase determines the redissolution of the precipitate.

Sedimentation as a Function of Temperature

The critical point in the curves is also in evidence if the temperatures are plotted as abscissae. In experiment 39 (Fig. 55) and experiments 41, 44 and 46, (Figs. 56 and 57) the upper curve corresponds to readings obtained with a serum which was heated and diluted (always by addition of five times its volume of water) without previous stirring, that is with the sediment at the bottom of the cell, whereas the lower curve corresponds to the same solutions but stirred so as to bring the sediment back into suspension. The curves meet 24 hours after the dilution, when the serum has been heated ten minutes at 57°, which signifies that there has been no sedimentation. This point corresponds to a stable suspension of globulins.

Influence of the Duration of Heat

Until now we have considered only sera heated during 10 minutes. When the heating is prolonged at a constant temperature, the point which corresponds to the meeting of the curves (*critical point of the stable suspension of globulins*) is shifted toward the lower temperatures (Figs. 55, 56, and 57). For horse serum, for instance, after two hours of heating, this point is at 53° (in one series of experiments). For one hour of heating it is at 54° and for five minutes at 58°. Fig. 58 shows the general aspect of the phenomenon of shift of the critical point.

When the precipitate is left in contact with the solution for three days (instead of 24 hours) it undergoes an alteration and does not entirely redissolve when NaCl is added.

One can try to find out experimentally if this critical point—around 57° for 10 minutes' heating—corresponds to a change in the state of dispersion of the globulins. In order to do this, the factor of depolarization, which was studied in the preceding chapter, can be used.[3] The reader is reminded that

[3] See page 64.

the factor of depolarization (ρ) is equal to the ratio $\frac{I_h}{I_v}$ of the light beam horizontally polarized, to the flux vertically polarized, laterally scattered by a liquid or a solution. ρ is always comprised between 0 and 1, and the less vertically polarized light there is in the scattered beam, the higher its value. As we have seen, the ratio ρ depends, among other factors, on the molecular optical anisotropy. Ultramicroscopic colloidal particles are gen-

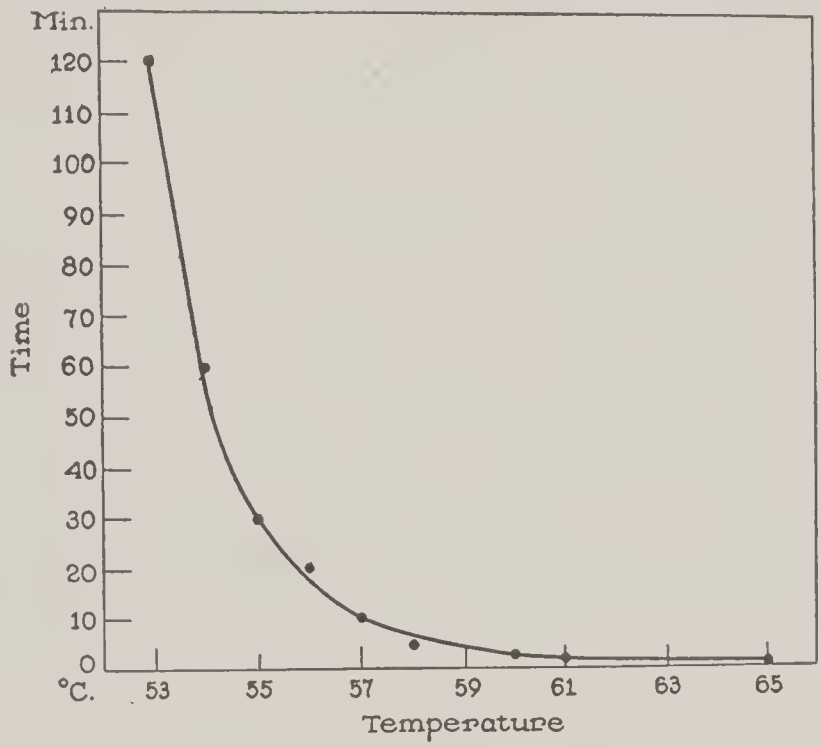

Fig. 58. Curve expressing for a normal horse serum the shift of the point (critical point of stability) at which curves like those of Figs. 55, 56, 57, meet, but for sera heated during different lengths of time. It can be seen that the influence of the time of heating is far greater above 55° C. than below this temperature.

erally spherical and isotropic, and tend to decrease the value of ρ. A liquid which diffuses an almost completely polarized light is a colloidal solution and not a homogeneous liquid.

We have shown that neither undiluted serum nor serum diluted with a physiological solution behaved like a colloidal solution, but on the contrary like a true solution, and that ρ increased considerably when the serum was heated. This indicated that the optical anisotropy of the molecules increased as a result of hydration.

Now we have just seen that, between 57° and 58°, there is an important modification in a serum which has been heated and then sufficiently diluted with water so that some of its molecules precipitate. Below 57° they sediment; above 58° they stay in suspension. The aspect of the solution is colloidal, or milky; but does this solution really attain a state of colloidal

dispersion between 58° and 60°? Or does this aspect, as is the case with undiluted serum, correspond to a hydration of independent molecules?

A glance at Fig. 59 shows that an important drop in the factor of depolarization corresponds to temperatures around 58°; this can tentatively be attributed to the formation of isotropic colloidal granules. We have never observed a similar drop in the case of undiluted serum or serum diluted with

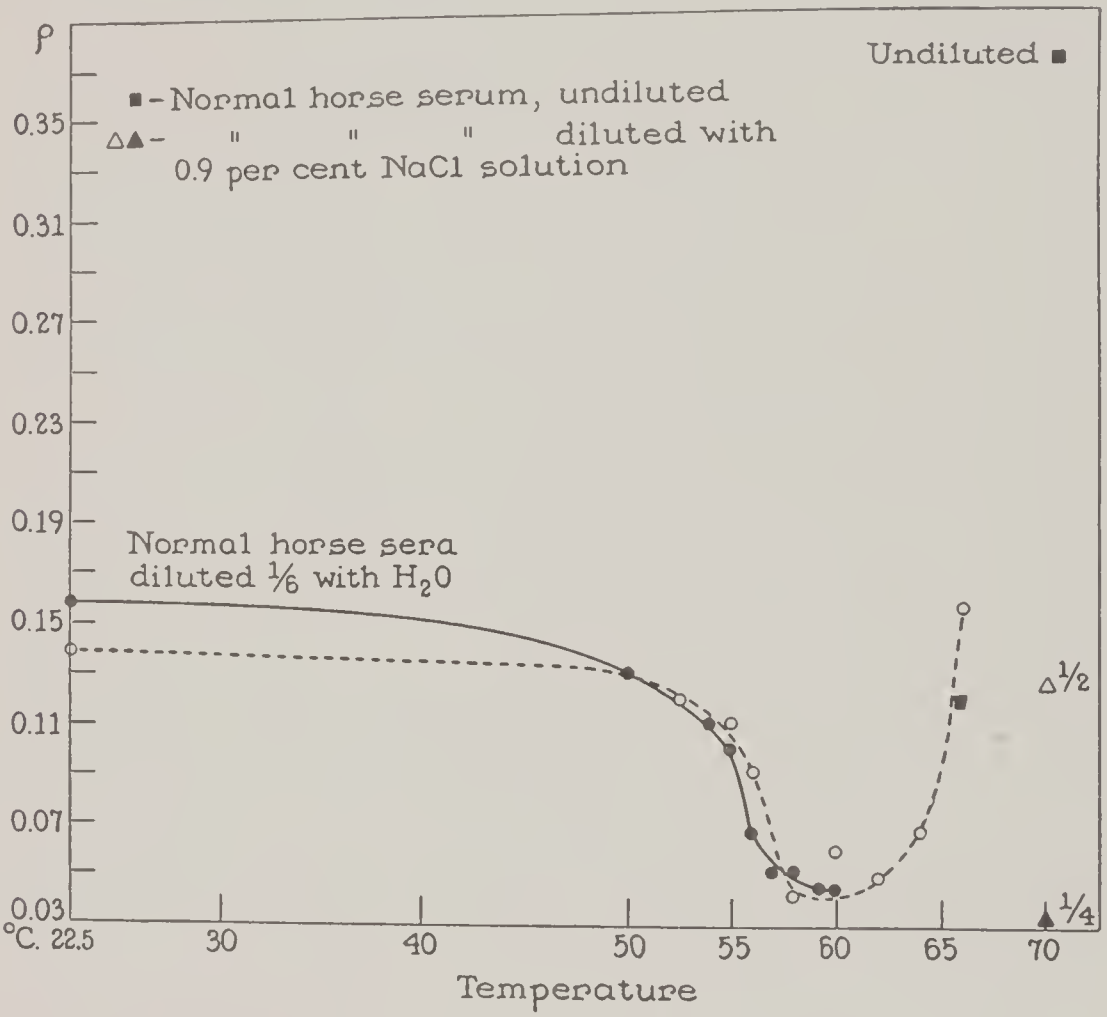

Fig. 59. Depolarization factor of horse serum solutions as a function of temperature. An important decrease can be observed above 55° C. This decrease is not observed when the serum is diluted with a 0.9 per cent NaCl physiological solution. The black and white triangles correspond to the values obtained with a serum diluted in this way to $\frac{1}{4}$ and $\frac{1}{2}$. It may be noted that these values are lower than those observed with serum diluted to $\frac{1}{6}$ with distilled water.

a salt solution. On the other hand, the initial value of the factor of the serum *solution* (unheated) is much higher than that of undiluted serum. The hypothesis can be made that this high value is due to the modification introduced in the optical anisotropy of the molecule by dilution of the salts.

In any case, for undiluted serum there is not a decrease, but on the contrary an increase of this anisotropy, which means that the solution is not

colloidal; whereas in the case of serum diluted with distilled water, the drop in the value of ρ, which is sometimes considerable, can be explained by the momentary aggregation of particles.

But when a certain minimum value has been reached (below 60° for horse serum, above 60° for ox and dog sera) a rapid and important increase of ρ is observed. This phenomenon is very difficult to interpret because of the double alteration to which the proteins are subjected, by dilution on one hand, and by high temperature on the other. Albumins and globulins are irretrievably denatured and probably disintegrated.

Thus, to summarize this chapter: when unheated serum is diluted with distilled water and heated to the critical temperature, *i.e.*, between 55° and 59°, according to the animal, a very clear modification of the dispersion of the precipitated globulins occurs. A difference in temperature of only one degree (between 57° and 58° for instance) is sufficient to bring about this modification, which manifests itself by a stability of the suspension at 58°, whereas it is sedimented at 57°. The sharpness of the phenomenon depends among other factors on the pH of the solution.

There is a *critical point of stability* which is displaced toward the lower temperatures when the time of heating is increased. For horse serum, for instance, this takes place at 58° for five minutes' heating and at 53° for two hours' heating. The influence of the time of heating decreases very rapidly below 55°.

The factor of depolarization of diluted serum, which is high when the serum has not been heated, decreases suddenly around 55° and reaches a minimum value around 58° for horse serum and around 65° for dog and ox sera, after which it increases rapidly. The stability of the solution (no sedimentation, or very slight sedimentation) seems to correspond to this minimum, which may characterize a colloidal state of the globulin solution.

Chapter 8

Ionic Phenomena of Serum as a Function of Temperature. II. Electric Conductivity

The preceding chapters already give a clearer idea of the nature of the physical phenomena which accompany and determine the particular phenomenon known as "destruction of the complement."

Logically, it was possible to foresee that another phenomenon could be added to those which characterize this critical temperature, *i.e.*, a modification in the electric conductivity of serum. Indeed, we had observed an increase in the volume of the molecules and a corresponding decrease in the volume of the solvent. The space available to the free ions is, therefore, reduced. The movements of the ions might be slowed down by the increase in the volume of the "serum molecules" which, as we have seen, manifests itself by an increase in viscosity. On the other hand, if only water molecules are adsorbed, the concentration in salts may be increased. Either way, it was plausible to suppose that a new characteristic manifestation of the critical temperature might be found. It was with this end in view that we undertook the experiments which will now be described.

First of all it was necessary to modify slightly the classical set-up of Kohlrausch, and we adopted the following technique.[1]

Technique.

(1) Source of alternating current. To obtain a sharply defined zone of silence, it is essential that the source of the current be as free as possible of harmonics, that is to say an almost perfect sinusoid. This result can be obtained, nowadays, by means of electron tubes, but the apparatus is rather complicated and we obtained excellent results with an oscillator constructed by the General Radio Corporation of Boston, which incorporates an electrically driven tuning-fork, a microphone, and an induction circuit. A six-volt direct current is thus transformed into an alternating current of sufficiently pure musical frequency (1,000 periods per second). It functions silently and very regularly.

[1] Lecomte du Noüy, *Ann. Inst. Pasteur*, **50**, 127 (1933).

(2) Audition. It is well known that the electrical balance of the arms of the Kohlrausch bridge is generally determined by means of a telephone. The drawback of this setup is that the operator is connected to the bridge by a wire and has to wear a head-phone which becomes very annoying if the measurements last any length of time. We therefore replaced the telephone by a loud speaker connected to an amplifying system with two electron tubes, directly connected to the telephone binding posts of the bridge. This set-up has several advantages. (1) The current which passes through the cell containing the liquid to be studied can be reduced to a minimum; this decreases the rise in temperature, without rendering the sound inaudible, as it is amplified about 800 times. (2) The operator remains independent of the apparatus, which makes the measurements less fatiguing. (3) The sensitivity of the bridge is increased, as the intensity of the current coming out of the bridge can be varied within considerable limits, while the current circulating in the bridge and the cell remains constant.

(3) Bridge. We used a Leeds and Northrup non-inductive dial bridge. The absolute values of the resistances are correct to only 0.05 per cent. But as we were looking mainly for modifications of the resistance in relative values we were able to obtain a much greater relative precision.

(4) Cell. We tried a great many different cells. There are a number of good cells on the market but they require a greater quantity of liquid than was available. We therefore had to find a sensitive cell of small capacity. As these two qualities are contradictory, many trials were made before the two following models, with platinum electrodes sealed in the glass, were adopted. It is indeed absolutely essential for the platinum to be rigidly sealed in the glass. If this is not the case, it is difficult and even impossible to repeat the measurements. It is unnecessary to add that the glass must be perfectly neutral ("Pyrex," for example).

(A). Cell of small capacity (2 cc). Fig. 60 shows that the shape of this cell is classical. The sensitivity of such a cell depends largely on the distance between the inferior electrode and the bottom of the cell. This distance must be *at least* equal to 5 mm when the diameter of the cell is 10 mm and that of the electrode disks is from 7 to 8 mm. If it is reduced to 3 mm the cell becomes five times less sensitive as a result of the increased electric capacity.

(B). Cell of mean capacity (8 to 10 cc) with variable sensitivity. Fig. 61 gives the appearance of this cell, which proved very satisfactory. The glass spheres have an inside diameter of 20 mm and the electrodes, with a diameter of 10 and 12 mm, occupy an ideal place in the center, thus reducing to a minimum the effects due to capacity. They are easily cleaned, as the two spheres can be separated one from the other, being coupled by a ground joint. The sensitivity can be varied by adding a ground, intermediary

piece between the two spheres. Without this addition such a cell has a resistance of about 650 ohms for pure horse serum or for a 0.7 per cent KCl solution. As the ratio arm on the bridge indicated 1/10, and as 1/10th of an ohm could be detected by ear, the sensitivity of the cell attained 1.5 per 10,000, or 0.015 per cent. This sensitivity required a thermostat sensitive to at least 1/100th of a degree, for a difference of 2/100ths of a degree could be discerned by ear, and the current, though very feeble, could not pass for more than one minute without producing an elevation in the temperature which affected the measurements.

Under these conditions the errors due to a change in temperature were negligible, for, expressed in units of conductivity, a difference of 0.01° gave an error of only 0.000,002,6 mho/cm, or of the order of 0.01 per cent.

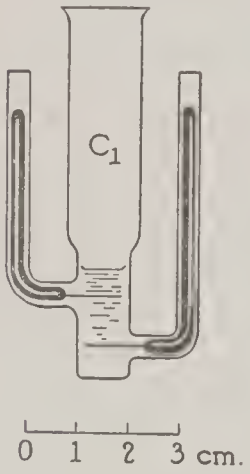

Fig. 60. Conductivity cell of small capacity C_1.

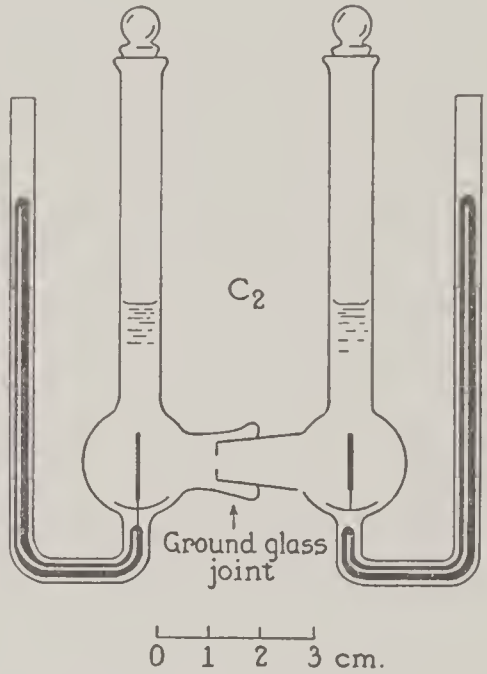

Fig. 61. Conductivity cell of mean capacity C.

(5) Thermostat. The electrically heated thermostat was a cylindrical vessel of enameled sheet iron, holding 20 liters, and insulated with a layer of felt 20 mm thick. The stirring was obtained by means of a screw-propeller with four blades, 12 cm in diameter, directly mounted on the prolonged main shaft of an electric motor. The screw-propeller was placed in the vessel laterally, at an angle of about 45°, so as to give the mass of water a gyratory motion. The motor was coupled to an auto-transformer or to a high resistance in series so that the rpm was 120, which insured complete and necessary silence. In this way, the stream of water pushed by the propeller hits the flat heating element (ordinary commercial water-heater) so that the water brushes it on both sides, thus insuring good calorific output

and rapid diffusion of the calories. The toluene and mercury regulator which was specially built for us[2] makes it possible to regulate to 2/1,000ths of a degree, when the periods of heating and cooling are long enough to bring about a thermal equilibrium between its mass and the water, and when the temperature of the laboratory varies little. Under the experimental conditions imposed by the relatively small mass of water and the imperfect insulation, we obtained a regulation of about ± 0.003° (periods of three or four minutes), which was satisfactory for our purpose. As the thermometer we used was graduated in 1/100ths of a degree, 2/1,000ths could be easily estimated with a magnifying glass.

General precautions. All the electric connections were electrostatically shielded and the shielding grounded. The oscillator generating the current was encased in a sheet-iron box constituting a Faraday cage, similarly grounded. Generally speaking, every precaution was taken to isolate each element electrostatically, and all these precautions combined enabled us to reach a sensitivity of 1/10,000 without difficulty. When needed, a variable air condenser was hooked up in parallel with the vessel, so as to balance the electrostatic capacity of the latter with respect to that of the bridge. We found experimentally that it was very advantageous *to ground one of the binding posts of the outgoing current* (A.C. of musical frequency connected to the cell).

Under these conditions the probable relative errors in the experiments reported here, do not exceed ± 0.026 per cent. The readings on the bridge express the resistance in ohms, readily transformed into resistivities by means of the constant of the vessel used. Resistivity is the resistance of a liquid cube of 1 cm, the section of which is, therefore, equal to 1 sq cm. But it is more usual to speak of *conductance* and of *conductivity*, which are equal to the reciprocal of the preceding magnitudes and are expressed in *mhos*: conductivity $= \frac{1}{\text{resistivity}}$ mhos/cm. We therefore publish the results of our experiments expressed as measurements of conductivity in mhos/cm.

All the above mentioned precautions were necessary, owing to the slight amplitude of the phenomena described in (C). This stands out in the figures.

We shall study: (A) The conductivity of normal horse serum, unheated, and then heated, as a function of the dilution. (B) The conductivity of undiluted and diluted serum as a function of the temperature, from 25° to 70°. (C) The conductivity of undiluted serum, unheated and heated for ten minutes, the measurements being taken after the serum had cooled to

[2] See: Lecomte du Noüy. "Méthodes Physiques en Biologie et en Médecine," pp. 36 and following, Paris, Baillière, 1933.

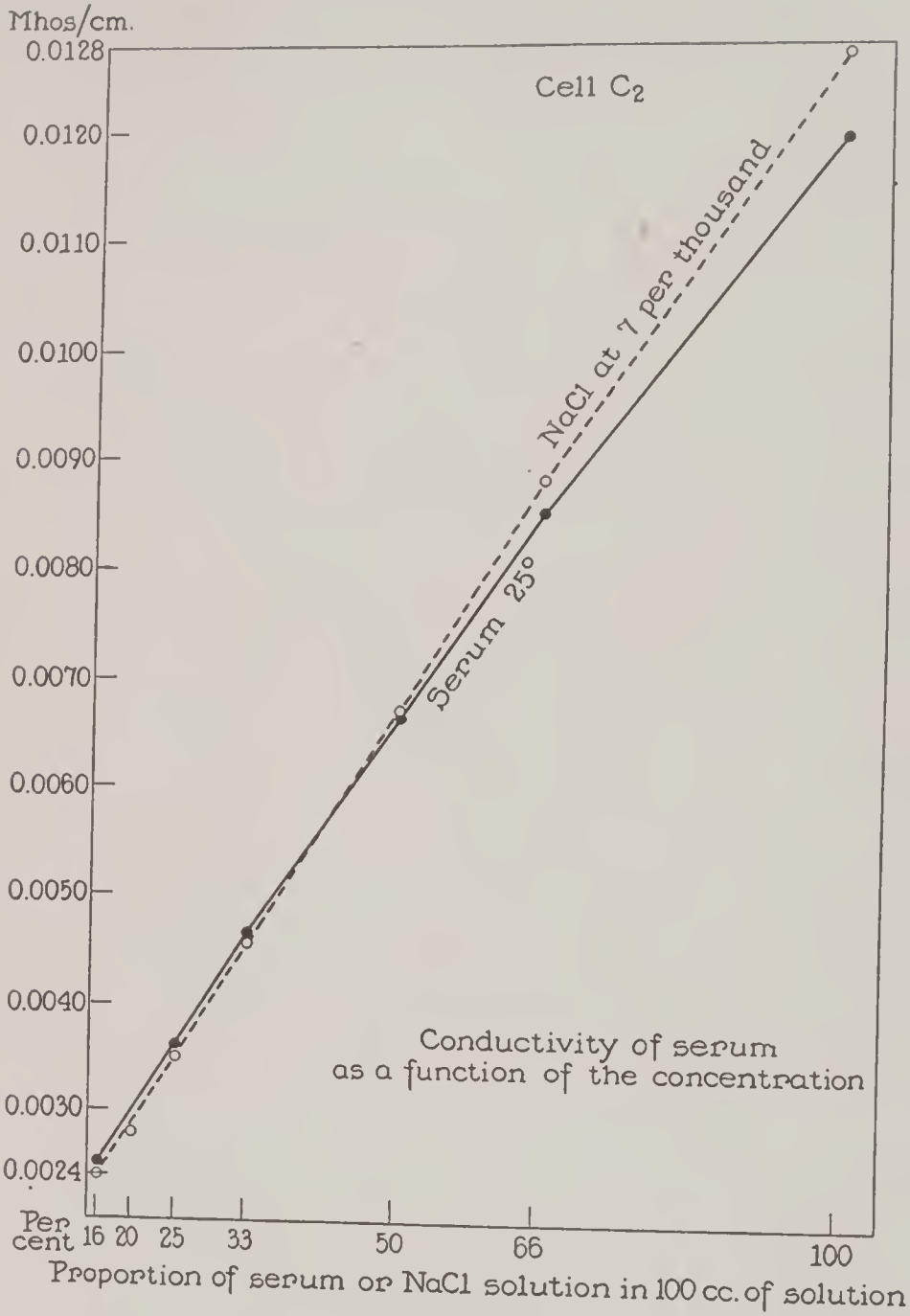

Fig. 62. Conductivity of normal horse serum at 25° C., as a function of the concentration and of a 7‰ NaCl solution diluted under the same conditions as the serum (distilled water).

25°. (D) The conductivity of serum diluted with distilled water after and before sedimentation of the precipitated globulins.

Experimental Results

(A) The conductivity of normal horse serum decreases progressively with the dilution, following a regular curve (Fig. 62) approximately identical with that representing the conductivity of a 0.7 per cent NaCl solution. It

can be seen that the conductivity of this sample undiluted is 0.01192 mho/cm at 25° ± 0.003, and 0.00245 mho/cm when diluted by addition of five times its volume of distilled water. When the serum was heated to 54°, 55°,

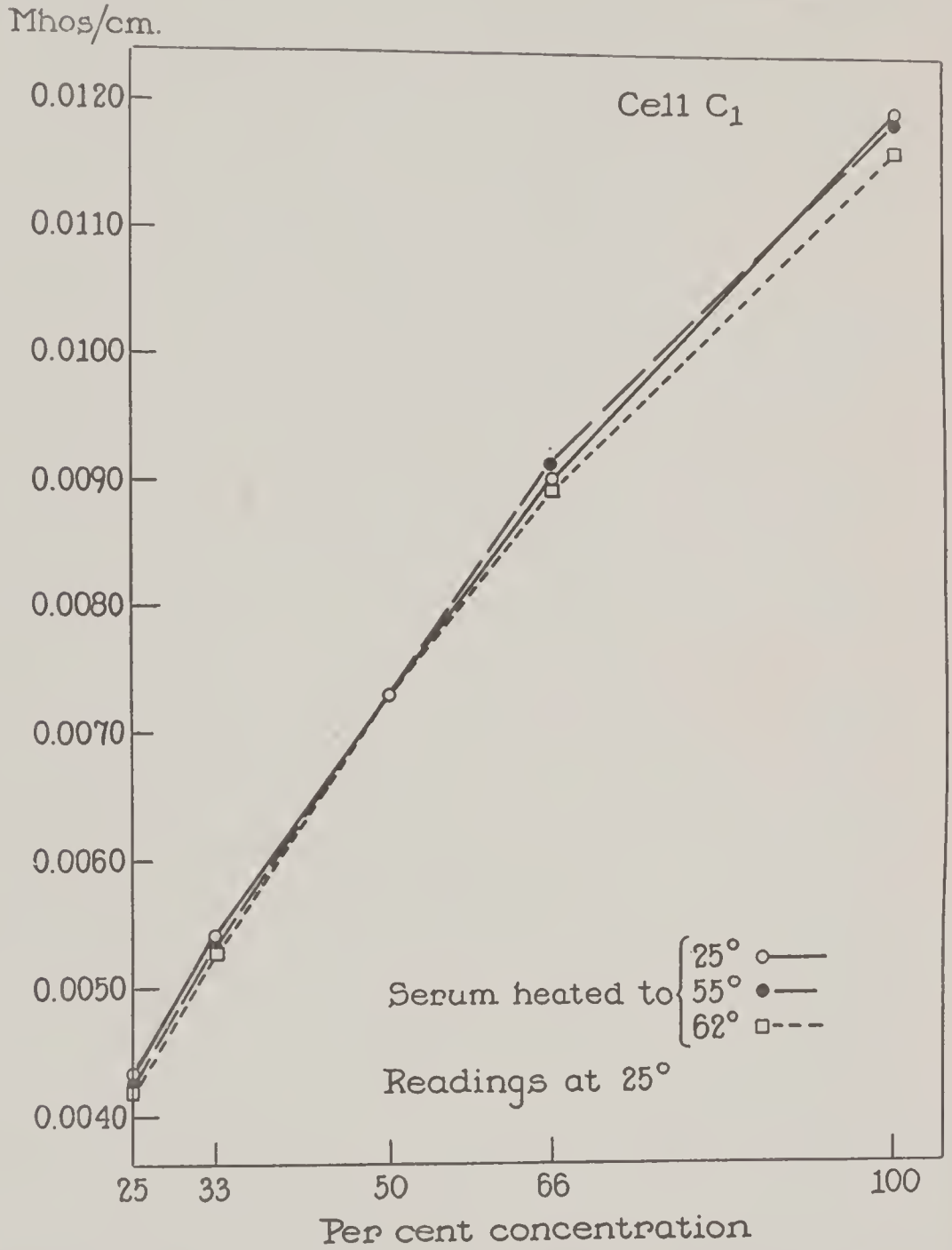

Fig. 63. Conductivity of a normal horse serum at 25° C., after having been heated to 55° and 62° C. for 10 minutes in a sealed tube, compared to the conductivity of the same serum unheated as a function of the concentration in distilled water.

58°, 59° and 62° in sealed tubes, then cooled and afterwards diluted, the curves representing the conductivity were very nearly identical with those obtained with unheated serum. The only difference was that heated serum had a tendency to show a *slightly* lower conductivity (Fig. 63).

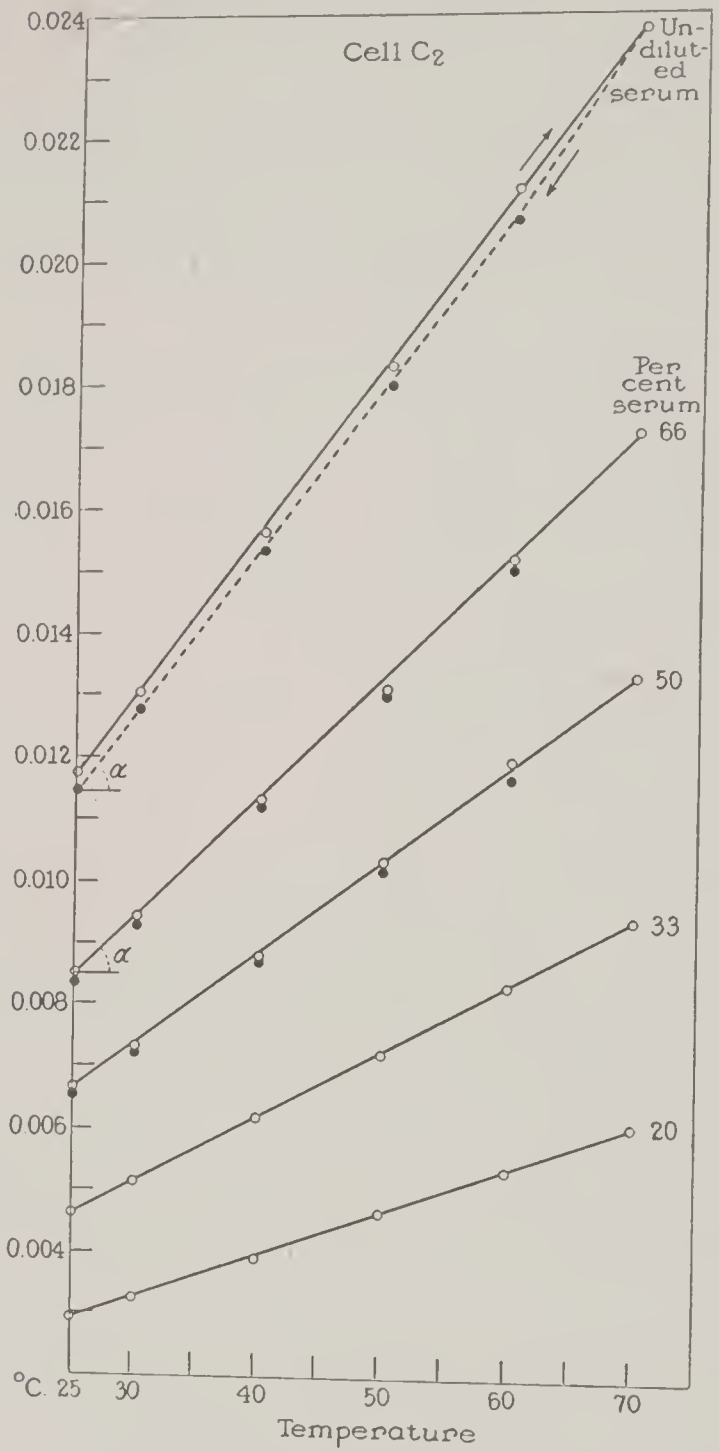

Fig. 64. Increase of the conductivity of undiluted serum and serum diluted in distilled water as a function of temperature. The dotted curve and the solid points express the values obtained during the cooling whereas the circles and the full curves correspond to measurements made during heating.

(B) The conductivity of serum increases almost linearly with the temperature, like that of a saline solution, but less rapidly. This means that the slope of the curve is a little less marked for the latter. Fig. 64 expresses this increase for undiluted serum and serum diluted with distilled water.

As the dilution increases, the angle of the straight line decreases proportionally to the dilution.

This stands out clearly in Fig. 65, where the tangent of the angle of these lines with the axis abscissae is plotted as ordinate and the percentage of serum as abscissa. This tends to prove that heating and hydration of proteins do not bring about fixation of ions on the latter but that they play only a restraining part. We shall discuss this later.

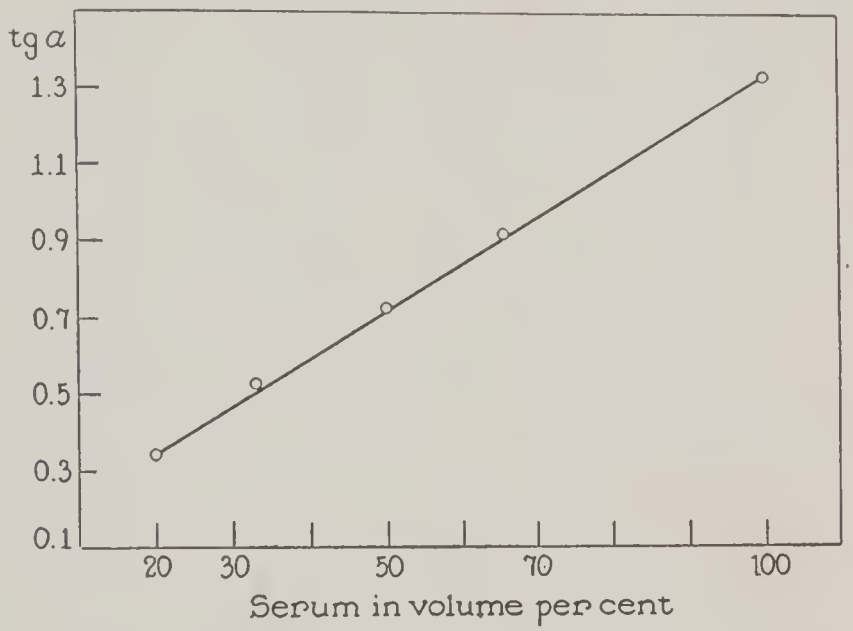

Fig. 65. The abscissae express the *tangents* of the angles of the Fig. 64 and the ordinates, the proportion of the serum in volume per cent. It can be seen that proportionality is rigorous.

(C) We now come to the conductivity of undiluted serum, heated for ten or twenty minutes in a sealed tube in a thermostat, and then cooled to the temperature of the laboratory. All the measurements were made at 25°

Table 26. Conductivity of a Normal Horse Serum (Institut Pasteur) Jan. 27, 1932. Cell C_2. Heated 20 Minutes, then Cooled to 25°.

Temperature (° C)	Conductivity (mhos/cm)
25 ± 0.003	0.013512
50	0.013512
56	0.013512
58	0.013526
60	0.013492
62	0.013454
64	0.013385
65	0.013385

± 0.003. Table 26 and Fig. 66 give the results of one experiment. The drop between 56° and 65° is 0.000,127 mho, or a little less than 1 per cent. A momentary increase of conductivity at 58° followed by an immediate drop is

observed. This accident often happens, but not always, sometimes at the beginning of the curve, sometimes later (see figures).

We therefore have another phenomenon, the sixth, to add to the list of irreversible and measurable phenomena brought about by the heating of serum above 55°. As we said in the beginning, this could be foreseen *a priori*.

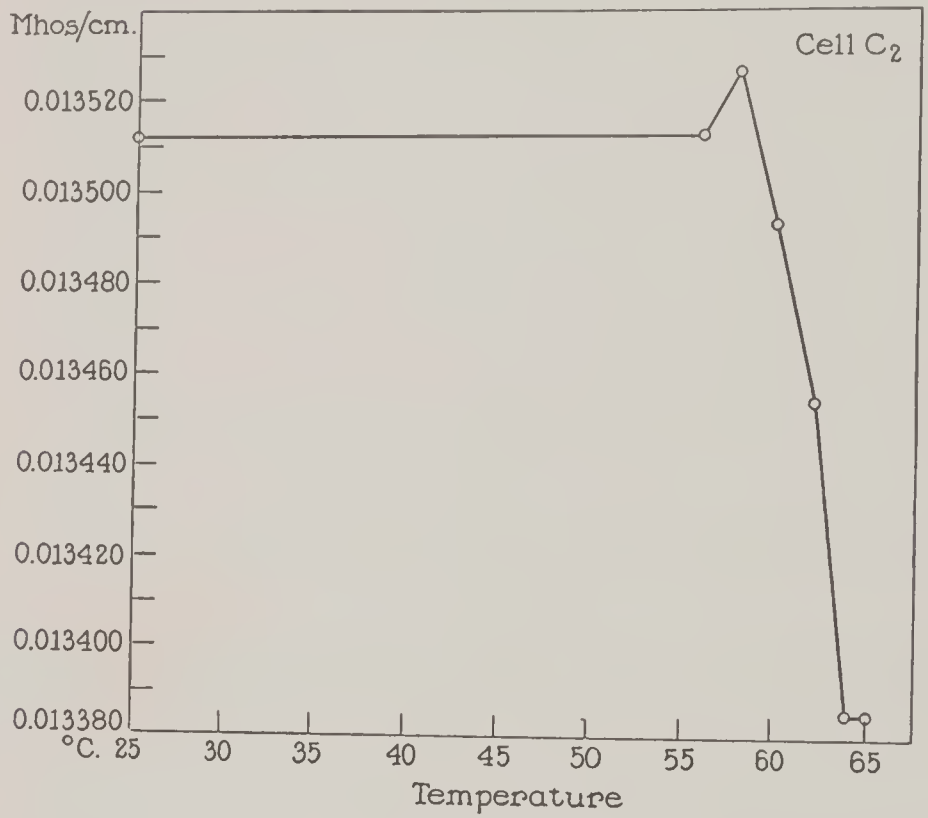

Fig. 66. Conductivity of a normal horse serum at 25° C., as a function of the temperatures to which it has been heated for 20 minutes.

Table 26 gives the results of an experiment with serum heated twenty minutes. The experiment in Table 27 was made with a serum heated ten minutes.

In the latter the drop is 0.000,076 mho, or about 0.6 per cent and the

Table 27. Conductivity of a Normal Horse Serum (Insitut Pasteur) April 27, 1932. Cell C_2. Heated 10 Minutes. Temperature of Measurement: 25°.

Temperature (° C)	Conductivity (mhos/cm)
25 ± 0.003	0.012168
50	0.012168
54	0.012168
55	0.012161
57	0.012151
58	0.012141
59	0.012103
60	0.012105
64	0.012092

phenomenon starts at 55°. For greater clearness we shall now replace the tables by curves. Figs. 67, 68, and 69 (phenomenon starting between 54° and 55°) show that the amplitude sometimes varies considerably, as serum No. 161 (Fig. 68) has a drop of 0.000,178 mho, or 1.5 per cent. They also

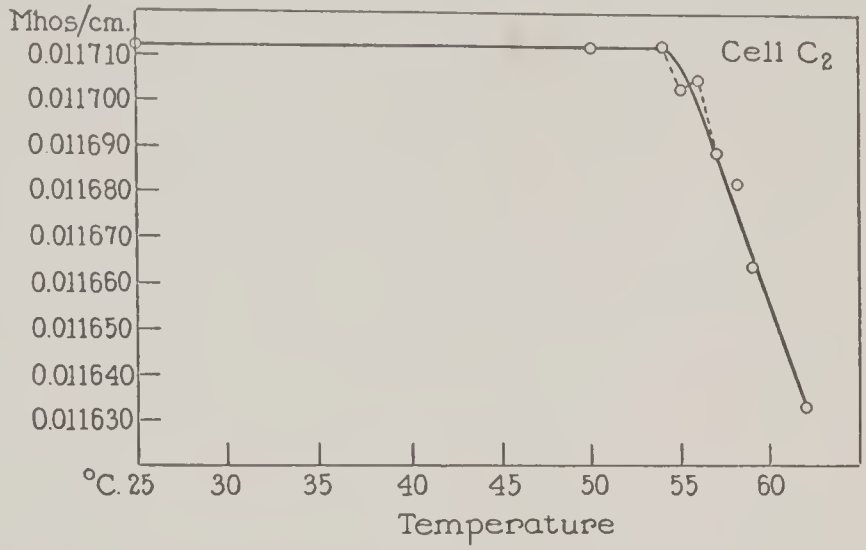

Fig. 67. Conductivity of a normal horse serum at 25° C., as a function of the temperatures to which it has been heated for 20 minutes.

show that accidents often occur in the curve, that is, that momentary increases of conductivity are sometimes observed which do not seem to be entirely due to experimental error. There is no change in this phenomenon of drop of conductivity when the serum is taken from an immunized

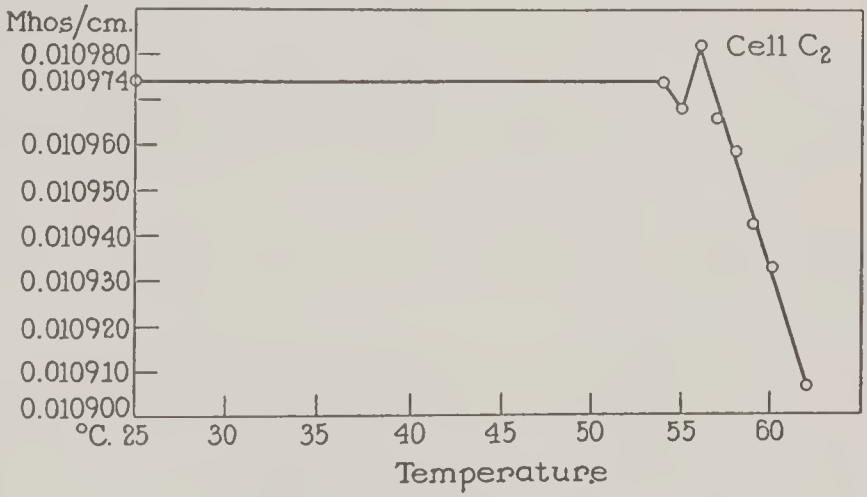

Fig. 68. Conductivity of a normal horse serum at 25° C., as a function of the temperatures to which it has been heated for 20 minutes.

animal. Fig. 70 (horse immunized against gangrene) shows this very clearly. We obtained the same results with animals immunized against perfringens, asp venom, and anthrax.

(D) When globulins have been precipitated with distilled water (5 cc of water + 1 cc of serum) and when measurements are taken of the resistance of the cloudy solution, after stirring, and of the clear solution decanted after the sedimentation of the globulins, it is found that the *clear solution* has a *lower resistance* than the cloudy solution and therefore *a higher conductivity*. The following figures, for instance, are obtained:

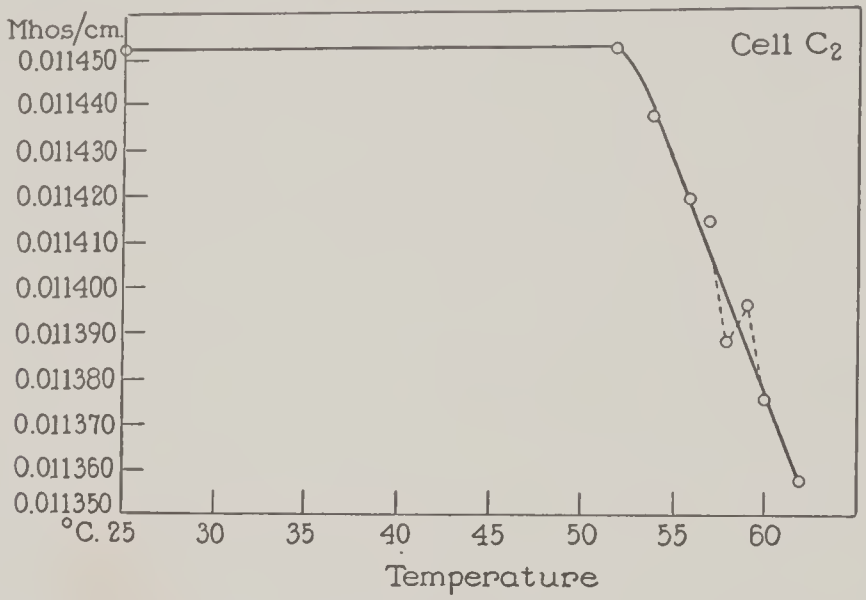

Fig. 69. Conductivity of a normal horse serum at 25° C., as a function of the temperatures to which it has been heated for 20 minutes.

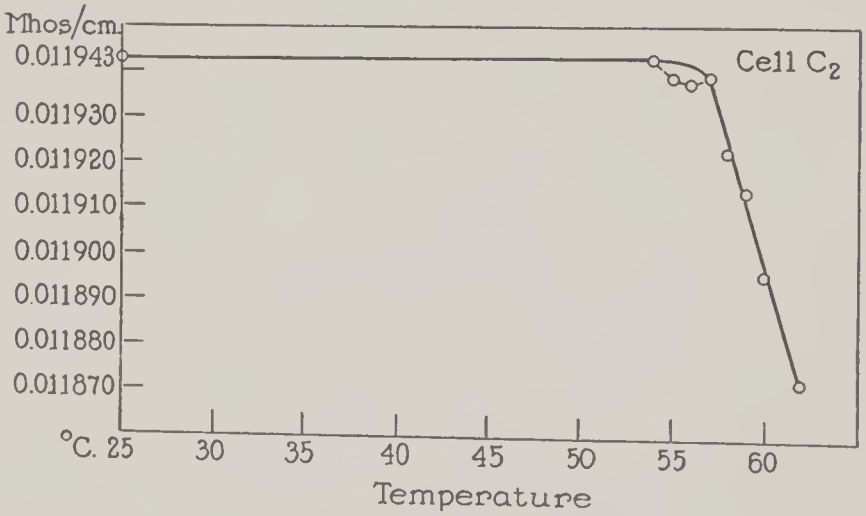

Fig. 70. Conductivity of an immunized horse serum (gangrene) at 25° C., as a function of the temperatures to which it has been heated during 20 mintues.

Exp. No. 1. Serum solution + H_2O (1/5) at the end of 24 hours. Stirred, cloudy liquid: resistance, 452.5 ohms. Clear, decanted liquid: resistance 448.4 ohms.

Exp. No. 3. Cloudy liquid: 459.4 ohms. Clear liquid: 450.7 ohms.

Conclusions

The explanation of this phenomenon of the increase of resistivity (or decrease of conductivity) is simple. The conductivity of a solution depends on the degree of dissociation of the electrolytes and on the friction of the ions. Now the non-electrolytes, the non-dissociated proteins of serum, on the contrary, act by slowing up the ions and perhaps, as Hamburger and others believe, by decreasing the dissociation. We say "perhaps," for the mechanism of this decrease does not seem clear.[3] Our experiments prove that the conductivity decreases after heating above 55°. Now we know that the viscosity of serum increases above this temperature as a result of the irreversible fixation of the water by the protein molecules. The slowing up of the ions is therefore increased, but only in a slight proportion, as a result of the decrease in the relative volume of the solvent and the increase in the volume of dissolved molecules. For instance, let us observe Fig. 69 and Table 27. We notice only a decrease of conductivity of 0.6 per cent in the table, whereas it is three times higher in Fig. 69, *i.e.*, 1.5 per cent. Now the increase in viscosity of the first serum was much greater than that of the second, which was almost coagulated at 62°, whereas, the first was not coagulated at 64°. This is, therefore, a mechanical phenomenon related to the hydration of the proteins. It should be possible to demonstrate this fact mathematically for, even if we cannot express it quantitatively, we possess elements which enable us to foresee the direction in which the phenomenon should act (increase or decrease of conductivity) and its order of magnitude. This calculation is useful; for, starting from the basic hypothesis, reasoning alone cannot foresee whether there will be an increase or a decrease.

Indeed, what happens when serum has been heated above 55°? First, water is fixed. This is an undisputable fact. But the fixation of water on

[3] The slowing up by the non-electrolytes decreases if the serum is diluted and the degree of dissociation increases. Oker-Blom [*Pflügers Arch.*, 79, 510 (1900)] calculated what he called the "physiological conductivity" of serum, which is nothing but the specific conductivity multiplied by the dilution. This physiological conductivity increases with the dilution, until it reaches an approximately constant value for a serum diluted 256 times. We have checked this. The pure saline solutions, without proteins, showing an identical initial conductivity, do not show as rapid an increase in conductivity, for only the dissociation enters into play. By a consideration of the observed values, Oker-Blom obtains a value for the dissociation constant of the salts in serum equal to 0.65. This figure is too low; Tangl obtains 0.82 by introducing a correction for friction. Now a 0.7 per cent NaCl solution, diluted 20 times, has a dissociation coefficient of 0.88. The slope of the dissociation curve of serum electrolytes is more marked than that of pure salts. [See Shields, *Zeitschr. f. Physik. Chemie*, 12, (1893). See also: Hamburger, "Osmotischer Druck und Ionen Lehre," 1, pp. 474 ff., Wiesbaden, J. F. Bergman, (1902), who attributes the phenomenon to the presence in serum of CO_3Na_2 which is dissociated by hydrolysis into NaOH and CO_3NaH.] In the course of this hydrolysis OH ions are produced, the velocity of which is three times greater than that of most other ions; this would bring about an increase in the physiological conductivity.

the proteins brings about an increase in the concentration of the salts as a result of the decrease of the volume of the solvent, and consequently an increase in conductivity. On the other hand, increase in the volume of the non-electrolytic molecules, which is the consequence of this fixation of water (aside from any hypothesis concerning the way in which this water is fixed), brings about a "slowing of the ions," a decrease of the conductivity. *A priori*, it is impossible to know which of the two contradictory phenomena will overshadow the other, or whether they will annul each other if they happen to be equivalent. Now, by considering the experimental values reported previously, which express the quantity of water fixed on the serum proteins as a function of temperature,[4] we were able, with the help of P. Martignan, to calculate, on the one hand the increase of concentration of the salts and on the other the mean increase of the cross-section of the molecules. As far as the mechanism of slowing up is concerned, we were obliged to introduce some hypotheses intended to simplify the calculations. We arrived at the following conclusions: The increase of conductivity due to the increase of the concentration between 55° and 64° is slight with respect to the decrease of the conductivity due to the increase in the volume of the molecules. Therefore, qualitatively, the calculation foresees a decrease of the conductivity, which is confirmed by the experiments. But quantitatively the figure expressing the slowing up is about three times lower than the value found experimentally. The order of magnitude is therefore correct, but we do not yet possess all the elements which would enable us to calculate the observed phenomenon exactly.

The last experiments mentioned (D) clearly show the role of the particles of protein in suspension. In this case it is possible, moreover, that a certain quantity of the salts is carried into the sediment by absorption; but this cannot happen in the case of heating, where the molecules, swollen by water, stay in solution.

[4] See Chapter 1.

Chapter 9

Ionic Phenomena as a Function of Temperature. III. Concentration of Hydrogen Ions

We thought it might be of interest to try to see whether or not a modification in the concentration of hydrogen ions accompanies the phenomena so far studied. This seemed plausible because of the increase in the rotatory power above 54° and because of the fact that, when serum is diluted, different phenomena take place which can be linked to the ionic equilibrium of the system. The variations in the rate of sedimentation of the globulins, as we have seen in the preceding chapter, are an indication that such may be the case.

Previous Publications. For the first time we find previous publications which must be taken into consideration. Since 1907, several workers have studied the changes in the concentration of hydrogen ions during the process of inactivation of serum. Unfortunately, they do not agree, and the absence of technical details in their papers makes it impossible to determine the sources of error. It is, however, probable that all those who found an alkalinization, neglected to take sufficient precautions, if any, to prevent the escape of CO_2. Lieberman and Lieberman,[1] for instance (1907) observed an important increase in hydroxyl ions in horse serum after thirty minutes' heating at 56° (pH 8.10 instead of 7.7). So did Tangl, whom they quote, and Seligmann (1908),[2] who employed the colorimetric method, which can hardly be called accurate. These authors did not state how they heated the serum.

In 1909 Michaelis and Rona[3] found, on the contrary, that there was no difference in the concentration of hydrogen ions, beyond experimental error, when serum was heated at 56° and 100° for thirty minutes. These excellent workers took all necessary precautions, and came to the conclusion that no change took place in the pH during denaturation, whether or not there was coagulation. From this they concluded that coagulation by heat cannot be linked to a scission of the protein molecule, which would liberate acid and basic groups as is the case under the influence of trypsin. We showed by a

[1] Lieberman, L. and P., *Arch. für Hygiene*, **62**, 315 (1907).
[2] Seligmann, E., *Biochem. Zeitschr.*, **10**, 430 (1908).
[3] Michaelis, L., and Rona, P., *Biochem. Zeitschr.*, **18**, 317 (1909).

different method that there was no aggregation during heating, and that no scission could be produced, as the number of molecules remains constant per unit of volume. Our experiments, therefore, check those of Michaelis and Rona. In the same paper, they established the buffer action of serum and found that it was better buffered against acids than against alkalies—a fact which we confirmed experimentally.

Davidsohn[4] studied the reaction of serum heated at 56°, 65°, 75° and 100°. He diluted the serum five or seven times with 0.8*N* NaCl to prevent coagulation. The heating was done in closed vessels. He also found that the differences between the pH of unheated serum and serum heated between 55° and 100° for thirty minutes are of the order of magnitude of experimental error.

Sörensen and Jurgensen[5] conclude that total coagulation of the proteins of serum by heating always gives rise to a decrease in the concentration of hydrogen ions in the medium. But they had previously determined the optimum pH of serum for coagulation, about pH 4.70, and had worked on serum acidified to this point. In our opinion this detracts from the interest of their work.

Finally, Hugo Hecht[6] declares that alkalinity is increased during the inactivation of serum by heat, but he does not describe his technique nor does he give any experimental data. It is therefore evident that the question, although studied, has not been solved satisfactorily.

Experimental. A first set of experiments performed with different classical electrodes available on the market, including the hydrogen, quinhydrone and glass types, soon revealed the fact that no systematic change appeared in the serum as a consequence of heating for a short time at or around 56° to 57°. However, with the best American-made hydrogen electrode (Clark), a slight drop was occasionally observed. This could be attributed either to an accident or to the fact that the technique was not sufficiently reliable. We chose the second hypothesis, and momentarily discarding our main problem, we attempted to improve the existing methods for measuring the pH.

The main requirements which directed our efforts were:

(1) Great accuracy and reliability.

(2) Great sensitivity.

(3) Possibility of achieving the first two requirements with as small a quantity of liquid as possible.

(4) The greatest possible rapidity compatible with the three preceding re-

[4] Davidsohn, H., *Zeitschr. für Immunitätsforschung*, 5, 182 (1910).

[5] Sörensen, S. P. L., and Jurgensen, E., *Compt. Rend. des Travaux du Laboratoire de Carlsberg*, 10, 1 (1911-1913).

[6] Hecht, H., *Zeitschr. für Immunitätsforschung*, 36, 321 (1923).

quirements, so that a large number of measurements could be made without allowing the serum to undergo important changes as a consequence of aging.

It was decided to concentrate on the hydrogen-electrode method, for two reasons: (1) it is the only one which supplies absolute values and, therefore, does not depend for its accuracy on more or less stable and reliable buffer solutions; and (2) it is by far the most sensitive. Our main problem, then, was to try to make it more rapid, more economical, easier, and if possible still more accurate. These three points will be taken up successively.

Rapidity. The first hydrogen electrodes (Sörensen, Hildebrand) required a long time to reach equilibrium. It frequently happened that potentiometer readings were still going up 30 minutes after the liquid had been in contact with the platinized electrode; it sometimes took more than 40 minutes. After Hasselbach had shown that agitation materially reduced the time necessary to attain equilibrium, a certain number of excellent pieces of

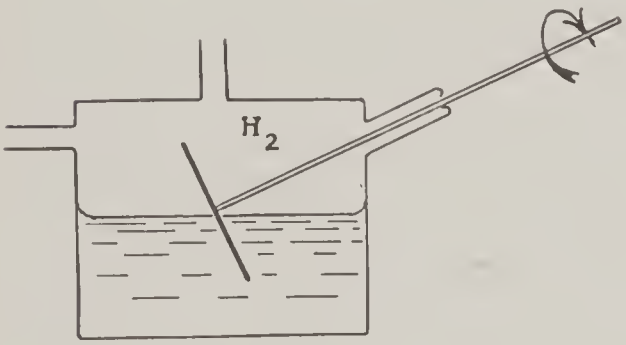

Fig. 71. Principle of the rotating tilted hydrogen electrode (du Noüy). The solid platinum disk at the end of the shaft is rotated by an electric motor and the readings made during the rotation.

apparatus were devised and placed on the market—the Clark model was definitely the best—and the time was cut down to about 20 minutes. Usually, the workers did not wait that long, as the rise in potential is so slow during the last 5 or 10 minutes that it affects only the second or third decimal place in pH values, and they thought they could neglect it and be satisfied with a pH value which was slightly, but systematically, too low. As a matter of fact, we found cases when the potentiometer readings were still going up after 30 minutes, at the rate of 0.1 mv in 3 or 4 minutes. As 0.1 mg corresponds approximately to 0.0017 in the scale of pH values, and as the third decimal figure is never, or almost never, taken into consideration, it seemed quite permissible to overlook differences as great as 0.4 or even 0.5 mv.

For all practical purposes, this is still true. But in our particular case, we were searching for a phenomenon which might conceivably affect the third decimal place. Therefore, we could not overlook even 0.1 mv and had to be certain that equilibrium was attained. Moreover, we found that: (1) certain samples of serum or plasma reached equilibrium much more rapidly than others, so that the error thus introduced was never constant; and (2)

that the time also depended on the platinization of the electrode. For these reasons, it seemed desirable to decrease the time required to attain equilibrium as much as possible.

This was done in the following way. A platinized platinum disk was mounted at the end of a shaft and tilted so that one-half of its surface dipped into the liquid (see Fig. 71). The rotation of the shaft brought every part of the disk successively into contact with the liquid, while a thin layer

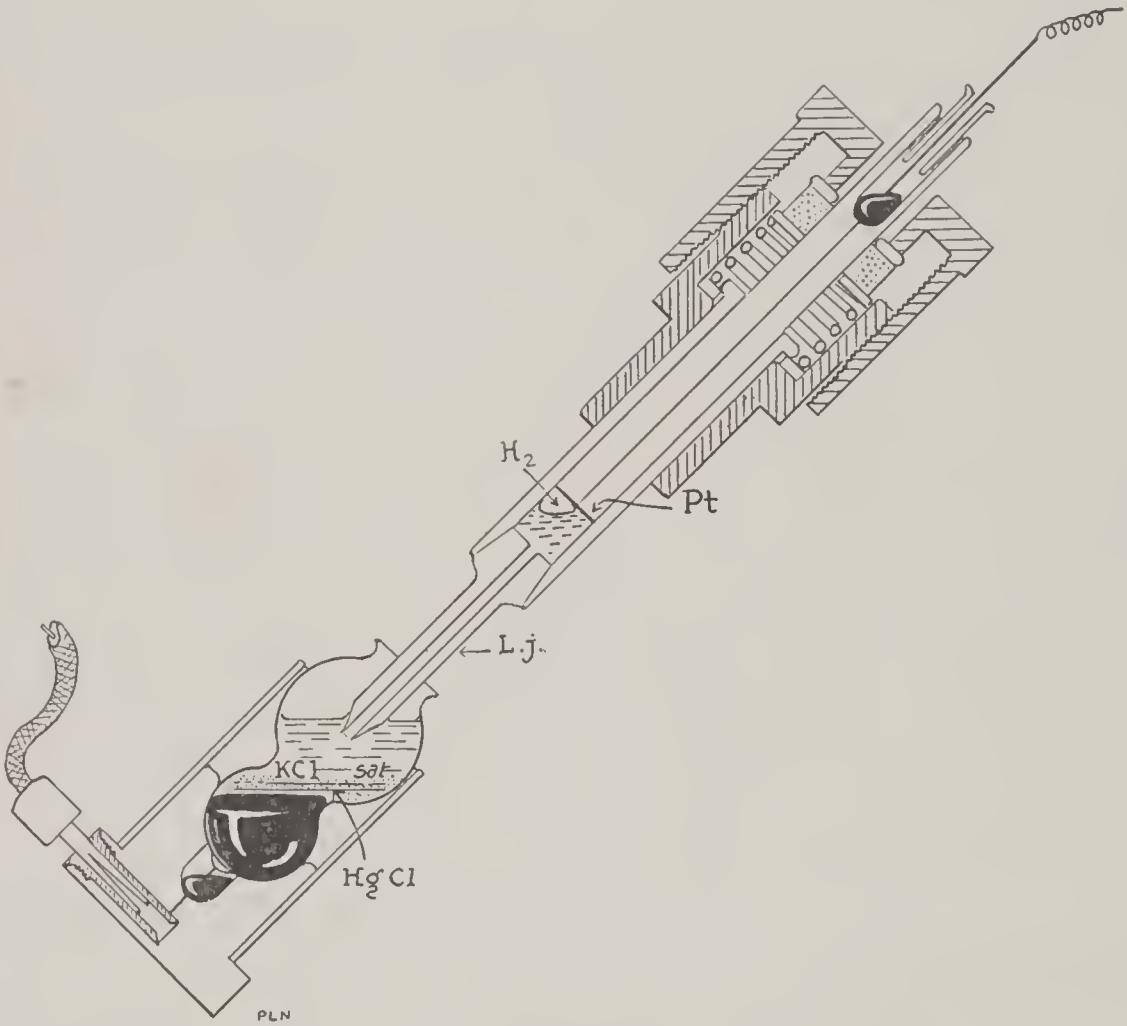

Fig. 72. Practical realization of the tilted electrode showing the saturated calomel electrode. The liquid junction is established in the capillary tip of the syringe. The piston has a platinum disk Pt., welded at its end.

of it clung to the platinum on its way out of the solution. As the whole was enclosed in such a way that hydrogen could be kept over the solution, and as the bearing of the shaft was gas-tight, the liquid raised by the revolving disk was, so to speak, sandwiched between the atomic hydrogen adsorbed on the platinum and the molecular hydrogen outside. Theoretically, the conditions were satisfactory. Practically, it was found that

equilibrium was reached in a much shorter time than with any previous set-up, namely, in a few minutes.[7]

Economy and Ease of Handling. In order to comply with the requirements mentioned above, the apparatus was finally constructed in the following way. A platinum disk was soldered to the end of the plunger of a "Pyrex" syringe (1 or 2 cc), thus acting as a piston. A platinum wire, passing through the plunger and emerging at the top, made the electrical contact possible from the outside by means of one drop of mercury. The lower end of the syringe was opened and ground so that a capillary tip could be quickly fixed or removed. Fig. 72 will suffice to show how the apparatus was set up.

It will be seen that instead of rotating the disk itself the whole syringe is kept tilted and in rotation around its axis. The hydrogen bubble, remaining on top of the liquid inside the syringe, is in permanent contact with

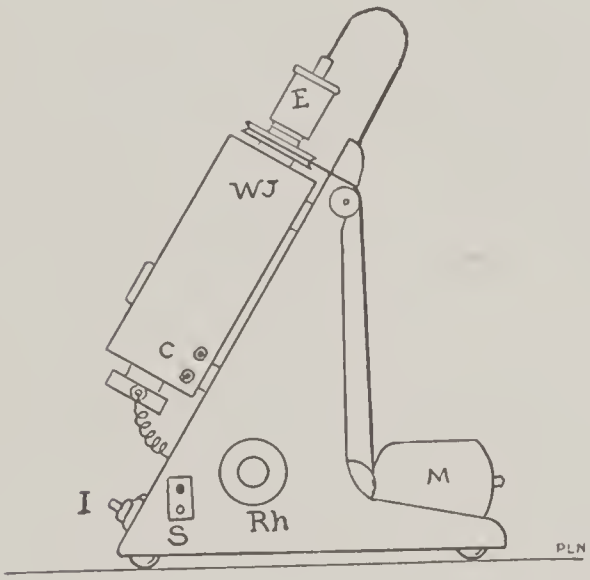

Fig. 73. General set-up of the du Noüy hydrogen electrode. The two half-electrodes are enclosed in a water-jacket (WJ). Water is circulated through the opening C. A polarized socket S permits an easy connection to the potentiometer. Rh is the rheostat for regulating the speed of the motor M, and I is the starting switch of the motor.

a constantly renewed film of liquid, thus duplicating the conditions achieved with the rotating disk.

The KCl of the saturated calomel half-electrode is aspirated half-way up the capillary tip; and as the liquid-liquid junction is of capillary dimensions (about 1 mm in diameter), practically no diffusion occurs. The syringe is fixed in a metal casing equipped with a screw top, and the motion of the plunger is accurately controlled by means of this screw.

The whole—calomel and hydrogen electrodes—is enclosed in a water-jacketed stand (Fig. 73). The metal casing holding the syringe slides in a tube mounted on ball bearings, and connected with a small electric motor in the base of the stand. The connections with the potentiometer are made through a polarized receptacle and plug, so that no mistake can be made.

[7] Lecomte du Noüy, *Compt. Rend. Acad. Sci.*, 193, 1117 (1931).

There are no glass stopcocks, rubber tubing, or other glass parts than those described. A rheostat controls the speed of the motor so that the number of revolutions of the electrode can be varied between 50 and 500 a minute.

Accuracy. In order to insure the highest possible accuracy, a systematic study of all the factors involved in a measurement was undertaken, namely: the platinization and hydrogenation of the platinum deposit, the purification of hydrogen, the volume of hydrogen, the volume of liquid, the rapidity and the time of rotation, and finally the equilibrium between partial pressures of the CO_2 contained in the serum and that of the CO_2 contained in the hydrogen. A technique was devised whereby the hydrogen used in every measurement was in rigorous equilibrium with the particular sample of serum studied. This had never been achieved before and was made possible by the small volume of hydrogen required (0.15 to 0.2 cc).

As a detailed report on this work has been published before[8] we shall not insist on the subject, and will simply mention the conclusion reached.

In order to obtain reliable figures expressing the pH value of plasma or serum with the above-described apparatus, accurate to ± 0.01 pH, and in the shortest possible time, it is necessary:

(1) To keep the temperature of both half-cells identical, and constant to ± 0.5°.

(2) To use 0.4 to 0.5 cc of serum or plasma and 0.15 to 0.2 cc of hydrogen.

(3) To saturate twice the hydrogen in the syringe with two samples of 0.4 cc of the same serum or plasma, by rotating the electrode each time for 3 minutes, discarding the liquid after the 3 minutes but keeping the hydrogen inside.

(4) To perform the measurements within 2 hours following collection of the blood. (Blood is collected, of course, under a thick layer of neutral paraffin oil.)

(5) To make sure that the platinization process is done under identical conditions of temperature, concentration of platinum chloride, density of current, and distance between electrodes. An automatic electrolyzer was specially built for this purpose and the solution was kept in a slowly rotating vessel, to insure homogeneity of the solution.

Under even better conditions (temperature constant within ± 0.1°, barometric pressure constant within 1 mm Hg), it has been possible to obtain with four different electrodes measurements which agreed to within ± 0.000,15 pH, with buffer solutions. Fig. 74 expresses the results of these measurements made in order to find the optimum volume of hydrogen to be used in the syringe. The measurements were made with the excellent acetate buffer solution of Michaelis. But in the case of serum or plasma, such

[8] Lecomte du Noüy, *Bull. Soc. Chim. Biol.*, 16, 177, Paris, 1934.

precision is entirely devoid of meaning, as the same sample will sometimes manifest spontaneous changes affecting the third decimal place. When dealing with biological, carbonated solutions, our experience has convinced us that to expect an approximation better than ± 0.005 is illusory.[9] This

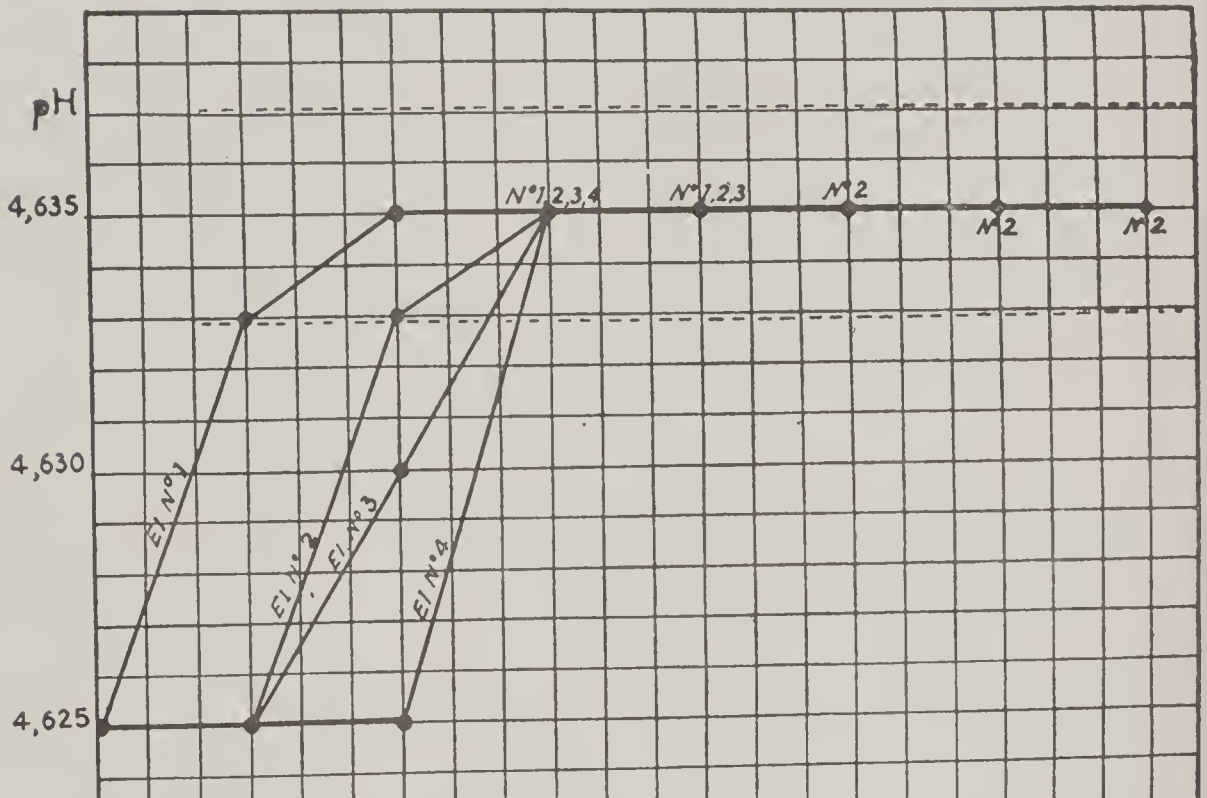

Fig. 74. Influence of the number of hydrogen bubbles introduced into the electrode-syringe.

is why it is in general unnecessary to take into account the barometric pressure, unless it changes by more than 10 mm, as such a change only brings about a difference in the pH value amounting to a little less than 0.003 pH. The figure mentioned above was given only as an illustration of the accuracy and reliability of the method.

[9] It may not be superfluous to remind the reader that the measurement known as the logarithmic index of Sörensen, or pH, is in reality a measurement of activity. Theoretically, it gives only an approximate value of the real concentration of H^+ ions. This last magnitude cannot be defined more accurately in our present state of knowledge, for even if the activity of an electrolyte in pure solution can be easily estimated, the problem becomes much more difficult to solve when the activity of each ion has to be considered, particularly in a solution containing several electrolytes. It seems reasonable to think that, in the case of such feeble concentrations as those of the H^+ ions in the organic liquids (10^{-5} to 10^{-8} n) the "activity" and the "concentration" are almost identical. However, this cannot be stated definitely on account of the very complex composition of the protein medium of the organism. The same restriction must be made for

Gathering and Conservation of Serum. It is known that the pH value of plasma, and in general of all carbonated solutions, depends on the tension of CO_2 above the solution, which determines the equilibrium (CO_2 — carbonates) in the solution. The mean partial pressure of CO_2 gas in the blood is about 40 mm Hg. Moreover, this pressure varies. That is why it is necessary, when making a measurement, to keep the solution or the serum in contact with a constant volume of hydrogen containing CO_2 at the same partial pressure as the liquid being studied. Under these conditions, loss of CO_2, which would profoundly modify the value of the pH, is impossible.

Therefore, when the pH of plasma is measured with the object of determining with the greatest possible exactitude the pH of the circulating blood (which is not perceptibly modified by the presence of figured elements), it is essential to gather the blood under neutral paraffin oil, to seal the centrifuging tube with paraffin melted at 50° and poured on the paraffin oil, and to remove the plasma or serum by means of a syringe thrust under the protecting layer. If these precautions are not taken there is a rapid alkalinization of the liquid due to loss of CO_2.

On the other hand, as serum is manipulated in contact with air when immunological reactions are made, and as the correlative physical phenomena of the destruction of alexin (increase of viscosity, of rotatory power, of electric resistivity, of scattered light, etc.), had been observed without any special precautions, we placed ourselves under the same imperfect conditions for certain experiments described later; that is, we did not keep the serum under oil.

Heating of Serum. The serum was always heated in sealed tubes under the same conditions as those described in the preceding experiments, and for certain accurate measurements, the free space above the liquid was reduced to a minimum. After heating, the tubes were cooled so that the CO_2 which had become separated during the heating could redissolve in the serum. The tubes were opened only at the time of the measurement.

For the heating we used a special water thermostat the temperature of which could be maintained at better than 0.5° for prolonged heating and within about 2/10ths of a degree for ten minutes' heating.

Filling of the Electrode. Saturation. All the measurements were made with 0.4 cc of liquid and 0.15 to 0.20 cc of hydrogen washed by passage

the measurements of the pH of solutions with a high saline concentration. In saline concentrations equal to or less than 0.15 *n*, that is, equal to or less than those of the liquids of the organism, the activity and the concentration of the H^+ ions do not show pH differences of more than + 0.01 to 0.02 (Kolthoff, 1925). "The order of magnitude of such differences in the pH reaches the limit of sensitivity of the methods of measurement of pH." (Bigwood, "Méthode de Détermination du pH des liquides de l'organisme," Paris, Masson, 1927). The method which we employ, however, has a much greater sensitivity, as we have shown. [Lecomte du Noüy and Hamon, *Bull. Soc. Chim. Biol.*, 16, 177, (1934).]

through a 5 per cent solution of pyrogallic acid alkalinized with potash. The hydrogen, under pressure, was forced through a fritted quartz filter immersed in this solution so as to obtain almost microscopic bubbles. The washing is thus very efficacious. The electrode syringe was always rinsed beforehand with the serum to be studied, and the hydrogen was saturated with CO_2 in the electrode-syringe, by rotation, in contact with two successive samples of the serum to be studied which were both eliminated and replaced by the definitive sample. The tension of CO_2 in the hydrogen was, therefore, determined by the tension of CO_2 in the very serum used for the experiment.[10].

Temperature. As the apparatus (Ionometer) was provided with water circulation completely surrounding both the hydrogen and the saturated calomel electrodes, all the measurements were made at a constant temperature, $\pm$ 0.5°. This temperature is always mentioned for every experiment.

Electric Measurements of Difference of Potential. For the positive half-element with respect to the hydrogen electrode we always used the calomel electrode with saturated KCl. We used the large model Type K Leeds and Northrup potentiometer, which is sensitive to 1/100th millivolt, coupled to the mirror-and-scale galvanometer made by the same firm. But we mostly employed an automatic recording potentiometer of Leeds and Northrup (Minimax), especially modified for us by the firm of M.E.C.I. in Paris. It had 33 sensitivities which enabled us to cover almost the whole scale of pH with the possibility of reading about 1/10th of a millivolt, *i.e.*, roughly, 2/1,000ths. These values, moreover, were frequently checked during the measurements with the standard Type K potentiometer.

The great advantage of this set-up combined with the rotating electrode, lies in the complete elimination of the individual coefficient in the measurements and in the possibility of obtaining recorded charts of all the experiments. Under these conditions the following results were obtained.

Experimental Results

First Group. For this group, no special precautions were taken in handling the serum; that is, it was manipulated in contact with the air and heated in sealed tubes. The free space over the liquid varied slightly from one tube to another.

Experiment No. 1: Normal Horse Serum: Temperature 20°. (Measurements taken with the recording potentiometer checked by the standard potentiometer and correct within 0.000,1 volt.)[11]

[10] See Lecomte du Noüy and Hamon, V., *Bull. Soc. Chim. Biol.*, 16, No. 2, 177 (1934) for all the details of the technique, and "Méthodes Physiques en Biologie et en Médecine," Paris, Baillière, 1933.

[11] It will be noticed that we have expressed the pH to the third decimal point in spite of the restrictions made above. The third decimal point must be considered only as an

In this experiment we find an increase in the pH value, that is, an alkalinization, as had been observed by a number of authors (Lieberman, Tangl, Seligmann, and Hecht).

Experiment No. 2. Normal Horse Serum: Temperature 20°. Time of heating 10 minutes.

Electrode No. 1		Electrode No. 2	
Temp. (° C)	pH	Temp. (° C)	pH
20	7.920	20	7.874
45	8.000	60	7.959
50	7.988	65	7.976
55	7.995	67	7.973

In this experiment alkalinization is much less marked and the phenomenon does not show a systematic increase. It is evident that there must be a disturbing factor independent of the measurement itself.

Experiment No. 3. Cock Serum: Temperature 20°. Heated 10 minutes.

Electrode No. 2		Electrode No. 3	
Temp. (° C)	pH	Temp. (° C)	pH
20	7.647	20	7.785
55	7.865	40	7.865
60	7.870	45	7.755
65	7.850	50	7.806
68	7.885		

This experiment is frankly bad. Even though alkalinization finally sets in, there are also unexplainable fluctuations.

Experiment No. 11. *Normal Horse Serum.* Manipulated in contact with air as in the preceding experiments, but the free space above the liquid in the sealed tubes was considerably reduced and was not greater than 0.2 cc, with slight variations from one tube to another.

Electrode No. 1		Electrode No. 3		Electrode No. 4	
Temp. (° C)	pH	Temp. (° C)	pH	Temp (° C)	pH
20	7.700	20	7.600	20	7.690
45	7.690	64	7.690	55	7.700
50	7.690	66	7.782		
60	7.660				

It is useless to publish the results of the 12 series of measurements made, for this last series (Exp. No. 11) clearly shows that: (1) The fluctuations

Electrode No.	pH				
	20°	50°	55°	60°	65°
1	7.920	....	....	7.940	....
2	7.906	....	....	....	7.965
3	7.906	7.933	....	....	....
4	7.920	....	7.935	....	....

indication of excess or deficiency and not as an absolute value. However, in the third group of experiments it will be seen that the precision is greater than the second decimal point, and that it can be taken into account as a relative value.

have become much less important, and (2) that the tendency toward alkalinization is almost suppressed (electrode No. 4) and even replaced by a slight indication in the opposite direction (electrodes 1 and 3). This indication suggests that the irregularities of the preceding experiments and perhaps even the alkalinization were due to loss of CO_2, which varied according to the amount of free space above the serum in the tubes. Therefore, a new series of experiments was undertaken in order to confirm or disprove this hypothesis.

Second Group. In this group the serum was manipulated in contact with air, but in sealed tubes with no, or very little, free space above the liquid. It will suffice to give the results of one series of experiments, performed with four different electrodes, so that in reality it represents four experiments.

Experiment No. 14. Normal Horse Serum: Temperature 20°.

Electrode No. 1

Temp. (° C)	pH
20	7.782
45	7.805

Electrode No. 2

Temp. (° C)	pH
20	7.787
50	7.805
55	7.795

Electrode No. 3

Temp. (° C)	pH
20	7.782
60	7.790

Electrode No. 4

Temp. (° C)	pH
20	7.798
63	7.765
66	7.795

These measurements show: (1) That when the free space is reduced to a minimum the fluctuations are clearly decreased. The differences between the values at 20° are slight (the three first electrodes give values which coincide to 5/1000. Only the fourth gives a value differing by 16/1000. (2) That it is impossible to state whether there is alkalinization or acidification, as the differences are too small or not constant.

This explains the differences in the results published by other authors and shows the necessity of manipulating the serum with rigorous precautions before the measurements.

Therefore, a third group of experiments was made, in which the serum was manipulated under neutral paraffin oil, and in which the tubes were sealed without leaving any free space above the liquid. The measurements were made with horse serum (Exps. 15 to 31, and 49 to 55), ox serum (Exps. 42 and 45), and sheep serum (Exps. 41 and 44).

Experiment No. 15

Electrode No.	pH					
	20°	45°	50°	55°	60°	63°
1	7.695	7.702	7.697	....	*7.650*	....
2	7.695	....	....	....	....	....
3	7.700	....	....	....	....	7.671
4	7.702	....	....	7.695	....	....

It can be seen that the values at 20° coincide for the four electrodes to better than ± 0.004, which is much more satisfactory and enables one, if necessary, to interchange the electrodes as the differences between the absolute values of the starting point are not greater than the admitted values of experimental error.

But the outstanding fact is the stability of the pH at all temperatures with the exception of 60°, when there is a difference of 0.045 pH toward acidity. This phenomenon will be found *in all the following series*, with occasionally a progressive decrease from 54° on.

Experiment No. 17

Electrode No.	pH						
	20°	45°	50°	58°	60°	62°	64°
1	7.815	7.805	....	....	....	*7.760*	....
2	7.833	....	7.822	....	7.790	....	....
3	7.815	....	....	7.795	....	....	7.790
4	7.833	....	....	....	....	....	....

In this experiment, the differences in the initial values were due to the calomel electrodes and not to the hydrogen electrodes. When the correction is made the difference is reduced to 0.008 pH.

Experiment No. 20. Normal Horse Serum: Heated 10 minutes.

Electrode No.	pH						
	20°	45°	50°	58°	60°	62°	64°
1	7.730	....	7.720	....	....	....	....
2	7.730	7.725	....	....	....	....	....
3	7.730	....	....	7.705	7.700	....	....
4	7.730	....	....	....	....	*7.685*	7.785

Experiment No. 23. Horse Serum: Heated 10 minutes.

Electrode No.	pH						
	20°	50°	55°	60°	62°	63°	64°
1	7.810	....	....	*7.757*	7.782	....	7.773
2	7.820	7.810	7.820	....	7.783	....	....
3	7.810	7.805	7.800	....	....	7.770	....

Experiment No. 26. Normal Horse Serum: Heated 10 minutes.

Electrode No.	pH							
	20°	53°	55°	57°	59°	61°	63°	65°
1	7.637	7.618	7.608	....	*7.564*	....	....	....
2	7.637	....	....	7.590	*7.564*	....	7.583	....
3	7.634	....	7.608	....	....	7.574	....	7.605

Experiment No. 27. Normal Horse Serum: Heated 10 minutes.

Electrode No.	pH							
	20°	54°	56°	58°	60°	61°	62°	64°
2	7.613	....	....	....	....	7.566	*7.545*	....
3	7.615	7.605	7.598	....	....	....	....	7.575
4	7.615	....	....	7.575	7.566	....	....	....

Experiment No. 49. Normal Horse Serum: Heated 10 minutes.

Electrode No.	pH								
	20°	54°	56°	58°	59°	60°	62°	64°	66°
1	7.730	....	7.702	....	....	7.692	....	....	....
2	7.725	7.725	7.718	....	....	....	7.682	....	....
3	7.730	....	....	7.696	7.692	....	....	*7.673*	....
4	7.730	....	....	....	7.685	....	....	....	7.677

Experiment No. 55. Normal Horse Serum: Heated 10 minutes.

Electrode No.	pH						
	20°	55°	57°	59°	61°	63°	65°
1	7.543	....	....	7.480	....	....	7.475
2	7.536	7.523	....	....	....	....	....
4	7.540	....	7.523	....	7.463	*7.445*	....

These two series of experiments show that the decrease in the value of the pH sometimes begins early, at 55° and at 56°. This is not frequent. It can also be seen that the *minimum* can be shifted up to 63° or 64°. Nevertheless, in the great majority of cases the minimum value is reached before 62°.

Experiment No. 42. Ox Serum: Heated 10 minutes.

Electrode No.	pH								
	20°	54°	56°	58°	60°	62°	64°	66°	68°
1	7.620	....	7.617	....	7.592	....	*7.550*	....	7.573
2	7.615	....	....	7.598	....	....	....	7.568	....
3	7.598	7.620	....	....	....	*7.550*	....	....	....
4	7.605	....	7.600	....	....	....	7.562	....	....

Experiment No. 44. Sheep Serum: Heated 10 minutes.

Electrode No.	pH							
	20°	56°	58°	60°	62°	64°	66°	68°
1	7.825	7.815	....	....	7.770	....	....	....
2	7.832	7.825	....	....	....	....	*7.748*	*7.742*
3	7.832	....	7.810	....	....	*7.748*	....	....
4	7.837	....	....	7.785	....	....	....	7.753

These data show that: (1) when the serum is manipulated under a layer of paraffin oil and kept from all contact with air, different samples of the same serum in different electrodes give values which are identical to 1/100. (2) With 10 minutes' heating there is *a minimum of pH, i.e.*, an *acidity*, usually between 59° and 62°, and that this slight acidification has a mean value of the order of 0.05 pH (the extremes being 0.03 and 0.07), which is much greater than the possible experimental errors. This acidity corresponds to a mean increase of about 13 per cent in the concentration of hydrogen ions. (3) An increase in the pH often follows this minimum when the temperature approaches that of coagulation (66°); ox and sheep sera gave the same results.

Influence of the Time of Heating. The time of heating does not seem to play an important part any more than in the physical phenomena previously described. After one hour of heating we sometimes observed the minimum at 58° that is, a little sooner; but the amplitude of the drop did not vary materially (0.071).

Experiment No. 39. Normal Horse Serum: Heated one hour.

	pH					
Electrode No.	20°	52°	56°	58°	60°	Coag. 62°
1	7.513	7.502	....	....	....	....
3	7.513	....	7.475	7.442	7.471	....
4	7.513	....	....	*7.451*	....	....

The phenomenon described above, *i.e.*, the slight acidification of heated serum between 58° and 62°, is affected by the initial pH value of the serum. In other words, if the serum is even slightly acidified *before heating*, to pH 6.80 for instance, there is very little or no decrease of pH after heating, as the following experiments will show. In certain cases there is even a slight alkalinization (Exps. P94 and P109).

Experiment P94. Normal Horse Serum.

Sample A (25 cc) acidified by a current of CO_2 for 8 min., then covered with paraffin oil. Heated 10 min. in sealed tube. Temperature of measure, 20°.

°C	pH
20°	6.710
60°	6.715
Difference	+ 0.005

Sample B (control) simply maintained under neutral oil, heated and measured under the same conditions.

°C	pH
20°	7.643
60°	7.580
Difference	− 0.063

Experiment P109. Horse Serum.

A. Acidified by current of CO_2 for 10 minutes. Heating and measurements as above.

(° C)	pH
20	6.760
60	6.764
Difference +	0.004
20	6.748
60	6.748
Difference	0.000

B. Control.

(° C)	pH
20	7.667
60	7.623
Difference	− 0.044
20	7.667
60	7.615
Difference	− 0.052

In order to study the phenomenon over a greater range of pH we acidified the serum by means of a normal hydrochloric acid solution in the following manner: 0.1, 0.2, 0.5 cc *N*HCl solution were added to 9.9, 9.8, 9.7, 9.5 cc of serum so as to bring the solution up to 10 cc. We were thus able to confirm the fact mentioned by Sörensen (loc. cit.) that a serum coagulates at 60°,

when its pH has been brought down to 4.78 (9.5 cc serum + 0.5 cc *N*HCl). We therefore had to acidify less. The serum no longer coagulated at 60° at a pH of about 5.4, but we found, as we mentioned above, that the heating determined a very slight alkalinization instead of the acidification observed with normal serum which had not been acidified beforehand. In other words, a serum heated for ten minutes at 60° and then cooled shows slight acidification or slight alkalinization (both of the order of 0.05 pH), *depending on whether the initial pH of the serum is alkaline or acid.* The curves join around pH 6.90 and there is neither acidification nor alkalinization; at least, if there is any it is of the order of magnitude of experimental error (Exp. P153).

Experiment P153.

		*N*HCl added (%)				
		1	2	3	4	
Control Serum (unheated)	7.49	6.90	6.38	5.98	5.38	pH
Serum heated at 60°	7.45	6.90	6.41	6.00	5.43	
Difference in pH	−0.04	0.00	+0.03	+0.02	+0.05	

The same phenomenon occurs with diluted serum, and the amplitude of the variations is of the same order of magnitude. As can be seen, the phenomenon is slight but constant.

Diluted Serum. As it was not easy to follow the alkalinization above 66° on account of coagulation, and as, on the other hand, it would be interesting to know if the minimum of pH was really followed by a rise, we diluted the serum with varying quantities of a 0.9 per cent physiological solution and with a solution of KCl at 9.525 per thousand.

Under these conditions we observed a more marked acidification beginning around 54°, but the increase of pH at temperatures above 62° was not observed regularly. The minimum was generally but not always reached at a higher temperature than in the undiluted serum and varied from one sample to another (Exps. 32, 33, and 50).

Experiment No. 32. Horse serum diluted with an equal volume of isotonic KCl.

	pH				
Electrode No.	20°	54°	60°	68°	70°
2	7.762	....	7.668	7.623	....
3	7.762	7.722	....	....	*7.595*

Experiment No. 33. Horse serum diluted with an equal volume of isotonic KCl.

Electrode No.	pH 20°	54°	56°	60°	62°	64°	66°	69°	72°
1	7.731	7.720	....	....	7.670	*7.655*	....	....	....
2	7.748	....	7.704	....	....	....	....	....	....
3	7.725	....	7.670	....	....	....	7.661	....	*7.655*
4	7.712	....	....	7.683	....	....	....	7.670	....

Experiment No. 50. Horse serum diluted to 1/8 (1 cc serum + 7 cc isotonic NaCl).

Electrode No.	pH 20°	55°	75°	85°	100°
1	7.635	....	*7,530*	....	....
3	7.635	7.633	....	7.555	7.566

It is impossible to reach a conclusion concerning what happens in undiluted serum above 62°, on the basis of the above experiments made with diluted serum and of many others which gave equivalent results. It can, therefore, be admitted that, when serum is heated around 60°, a minimum pH occurs which is sometimes followed by a rise toward alkalinity. The interpretation of this fact is very difficult for the time being.

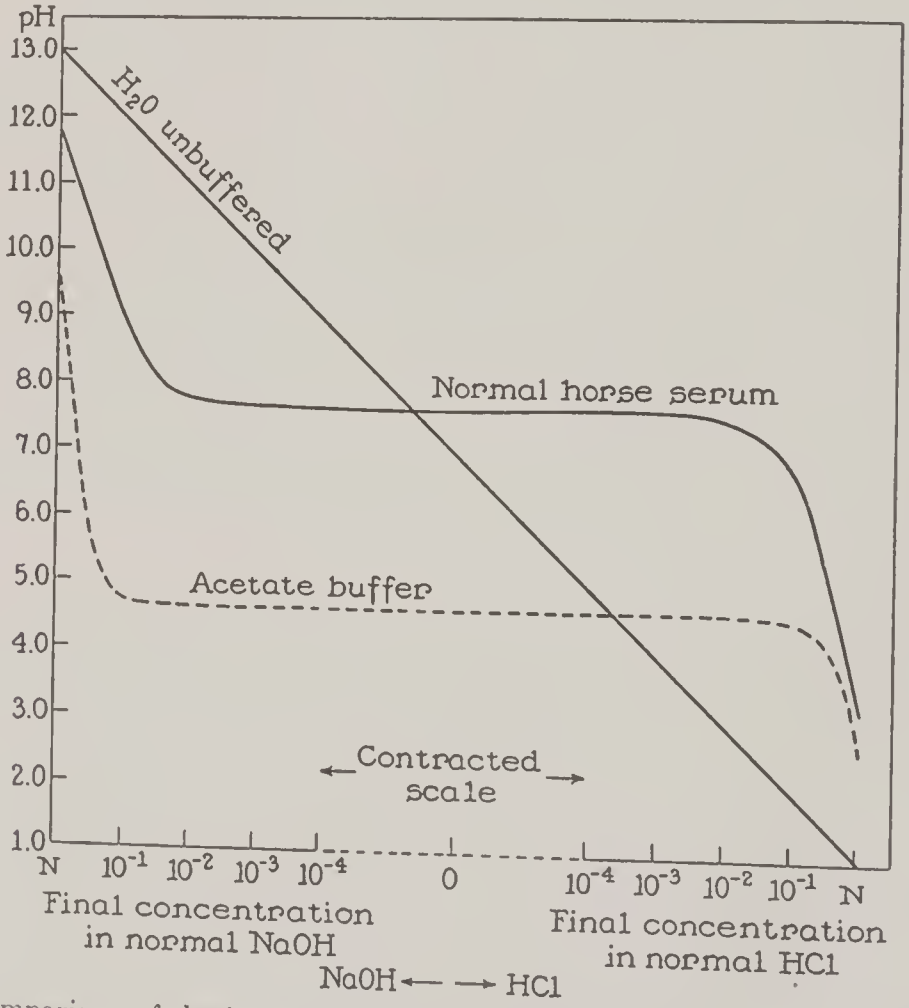

Fig. 75. Comparison of the buffer action of horse serum and that of the acetate buffer solution.

Buffer Action of Serum. We wondered if this acidification was not related to a decrease in the buffer action of serum as a result of heating. To answer this question a series of measurements were performed with heated and unheated sera to which varying quantities of HCl and NaOH had been added. The results of these experiments are expressed in Fig. 76. Fig. 75 shows the buffer action of the Michaelis solution next to that of heated serum; the diagonal line represents the value of water (buffer = 0) when the same quantities of acid or alkali have been added. Fig. 76 shows clearly that the buffer action of serum is not modified by heat. On the

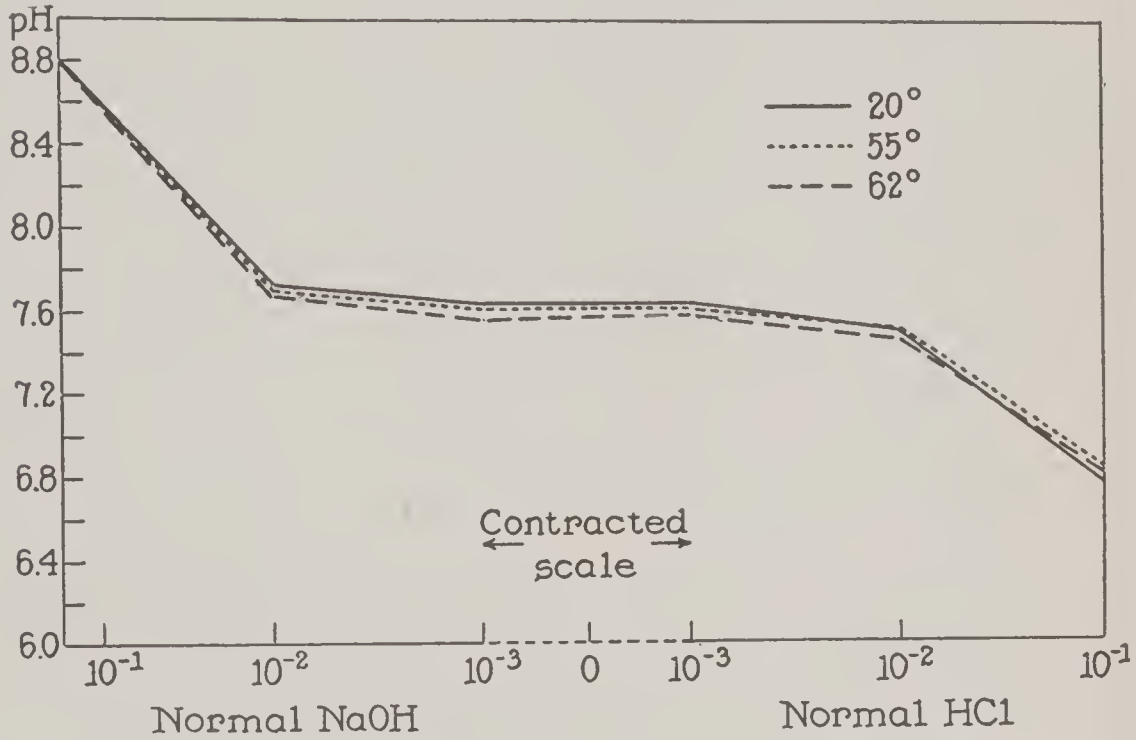

Fig. 76. Buffer action of unheated serum at 20° C., and serum heated for 10 minutes at 55° and 62° C. Concentration of the Na OH or HCl solutions added to 9cc. of serum in abscissae. (1cc.+9cc.=10)

other hand, one clearly sees the change due to heating which shifts the whole curve toward acidity. It can also be seen that the serum is better buffered on the acid side than on the alkaline. (Michaelis.)

In brief, when blood serum is manipulated with proper precautions ensuring a minimum loss of CO_2, and when it is heated for only ten minutes, a minimum pH is observed, that is, a maximum of acidity, around 60°. Up to 56° the pH value is approximately constant, but a beginning of acidification is often observed around or above this temperature.

This phenomenon is constant but of slight amplitude. The mean value of the drop is of 0.05. Beyond 60° a rise in pH often takes place (alkalinization), which brings its value back to the same level or even above the

original value. These phenomena can be observed only if there is no loss of CO_2 before, during, or after heating.

The alkalinization mentioned by certain authors was therefore due to the loss of CO_2, which they had not tried to avoid. Those who concluded that there was an absence of variation after heating employed a technique which was more rigorous, but their methods of measurement were either not sensitive enough or not accurate.

When the serum is diluted with isotonic solution the same phenomenon takes place. The drop is more pronounced, generally starts around 55°, and progressively reaches a minimum. Above 60° the pH values oscillate around the minimum value and rarely show a tendency toward alkalinization.

This phenomenon characterizes normal serum with a pH between 7.20 and 7.80 (at 20°). If the pH of the serum is lowered to about 6.80 or 6.90 before heating, the amplitude of the phenomenon decreases considerably and tends toward 0. When the acidity is higher (up to 5.4, for example) the phenomenon is inverted and an alkalinization can be observed when the serum is heated at 60°. The mean amplitude is the same as in the preceding phenomenon, *i.e.*, 0.05 pH. The curves representing the variations of the pH of two samples of the same serum—one heated to 60° and the other unheated—as a function of the quantity of acid added, therefore, cross each other around pH 6.85.

Heating at 62° does not modify the buffer action of serum. This phenomenon, *i.e.*, the slight decrease in the pH value of the serum, usually followed by a rise, can be added to the list of those which we have already described and which characterize the critical temperature of serum and the destruction of the alexin by heat.

Chapter 10

Fixation of Ether by Serum as a Function of Temperature. Parallelism with the "Complementary Power"

So far, the only connection between the phenomena we have described and the destruction of the complement is the existence of a common cause: heating at a certain temperature; but this connection sheds no light on the biological phenomenon itself. We observe simultaneously a certain number of phenomena, different in nature, as a consequence of a treatment of the serum involving only one variable. We logically conclude therefrom that a correlation exists between all the observed effects, and that the biological effect is linked to the chemical (structural), physical and physico-chemical phenomena by a relation of cause to effect. At this point all we can do is to propose a hypothesis which is plausible but not demonstrated. Its rigorous demonstration would be difficult. However, this hypothesis is the simplest that could be chosen to explain all the important physico-chemical and structural modifications which we have described. It would be quite incomprehensible if these profound alterations had no repercussion in the biological reactions, and there is too much similarity between the mythical existence of that elusive entity called the "complement" and the phlogiston for us not to make every effort to destroy it.

The only resource we had, therefore, was to try to find an ever increasing parallelism between certain physico-chemical phenomena and the biological phenomenon in the hope of shedding some light on the mechanism of the latter.

One of my collaborators at the Pasteur Institute in Paris, Dr. Franz Seelich, who was naturally working in the same direction, discovered a series of new phenomena which we shall describe briefly.[1] He observed that unheated normal serum was capable of fixing a considerable amount of ether (700 per cent) if the ether, $(C_2H_5)_2O$, was added in small quantities and the vessel was continually and energetically shaken. In such a case the serum became more and more viscous and finally solidified into a transparent

[1] Seelich, F., *Compt. Rend. Soc. Biol.*, 110, 794, 922 (1932); *Ann. Inst. Pasteur*, 52, 540 (1934); *Biochem. Zeitschr.*, 273, 135 (1934).

gel. No ether was fixed beyond 700 per cent. When agitated, a few extra drops determine a rupture of the gel into fragments with the exudation of a part of the ether previously fixed. The saturation point is quite sharply defined within 5 per cent. When the serum has thus fixed a maximum of ether, the gel is not stable and the exudation of the ether begins as soon as the agitation is stopped. After the elimination of the ether by exudation, it can be fixed again when added in small quantities. This is, therefore, a reversible and quantitative phenomenon. Serum heated to 50° reacts in the same way as unheated serum; but if it is heated for ten minutes at a

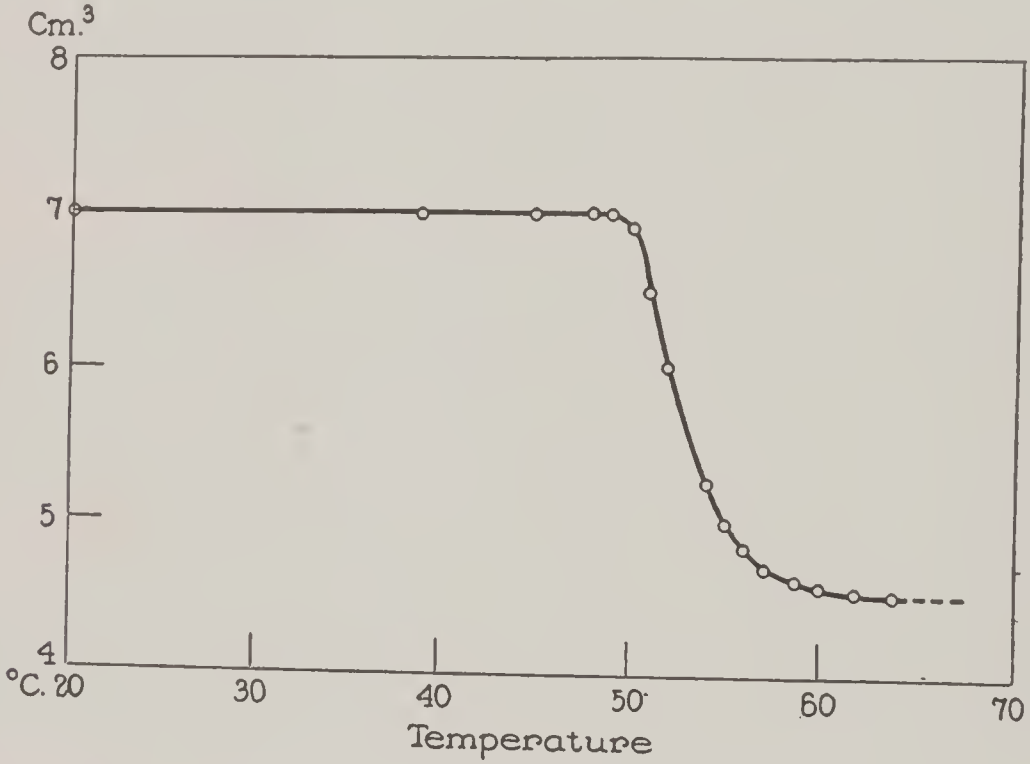

Fig. 77. Volume of ether (in cubic centimeters per cc. of serum) fixed by serum as a function of temperature.

temperature above 50° the quantity of ether which can be fixed decreases as a function of the temperature up to 58°, after which it remains constant. Serum heated at 58° fixes a quantity of ether about equal to $\frac{2}{3}$ of the quantity fixed by unheated serum, *i.e.*, 4.5 cc per cc, instead of 7 cc. Above 64° the values are no longer constant, probably because of the increase in viscosity. The general aspect of the phenomenon is shown in Fig. 77. The points of the curve express the mean values of three experiments.

This phenomenon is very remarkable because of the quantitative constancy of the results. One cc of serum always fixes the same quantity of ether within about 2 or 3 tenths of a cc. Seelich thinks that this is simply an emulsion. His experiments on the fixation of ether by solutions of bile

and sodium oleate salts (*Ann. Inst. Pasteur*, 1934) seem to confirm this point of view.

In his first paper he gave an explanation of this phenomenon based on the orientation of molecules, but his later work made him abandon this hypothesis. It would seem that these phenomena can be explained simply by what we know of unstable emulsions.

In further experiments Seelich observed that when unheated serum was diluted there was a slight increase in its total ability to fix ether. For instance, 0.5 cc of serum fixes 3.5 cc of ether. If 0.5 cc of water is added, 7 cc of ether will be fixed just as if 1 cc of serum had been used. But the increase of capacity due to 0.5 cc of water remains the same if it is added to 1 cc of serum, that is to say, 1 cc of serum + 0.5 cc of water will fix 7 + 3.5 = 10.5 cc of ether. Distilled water and NaCl physiological solution act in the same way. If 0.5 cc of water is added to 0.5 cc of serum previously heated (10 min at 57°), the same increase is observed, *i.e.*, 3.5 cc of ether, or 2.25 + 3.5 = 5.75 cc.

The question then arose as to whether the reaction would not be more marked if fresh serum were added. Experiments confirmed this hypothesis. The phenomenon in question is parallel to that of the destruction of alexin by heat, which is followed by "reactivation" when very slight quantities of fresh serum are added. In order to try to see how far this parallelism was carried, the quantity of fresh serum added was decreased, and it was observed that it suffices to add one drop (about 0.05 gr) of fresh serum to 1 cc of serum heated for 10 minutes at 57° to reactivate the serum almost totally, so that its capacity for fixing ether passes from 4.5 cc to a value very near or equal to 7 cc, (between 6.5 and 7.0). We therefore have here a new effect which is independent—above a certain minimum limit value—of the volume of serum added, and which seems to depend solely on the properties of the fresh serum.

The parallelism between the two phenomena—biological and physico-chemical—is quite clear. Unfortunately, until now the mechanism of the physico-chemical phenomenon is as little known as that of the biological one and cannot yet be used for an explanation.

These facts are very interesting. For the first time we possess a physical model which functions like the biochemical phenomenon. Seelich was also able to demonstrate that when a serum is heated for 10 minutes at a temperature above 50° (between 50° and 60°) and then treated with CO_2 (by letting a slight current of carbonic gas pass through for 10 minutes), there is an increase in the ether coefficient, whereas the ether coefficient of fresh serum does not change after this treatment.

It is evidently the change in the ionic equilibrium which brings about an increase in the power of emulsion. As the serum is buffered by carbonates,

phosphates and proteins, one can obtain a complete change in this equilibrium by making a slight change in the concentration of H^+ ions. But as we do not know the factors which underlie the mechanism of emulsification, it is impossible to specify the direct cause of the increase of the emulsifying power of heated serum as a function of the treatment described above.

We have shown that the heating of serum at a temperature which decreases or destroys the alexinic activity of serum also decreases its power of emulsion.

It was therefore interesting to find out whether the factors *which increase the emulsifying power of serum could not also increase the alexinic activity.* In the experiments made to verify this hypothesis we were obliged

Table 28. Different Saline Solutions Used to Dilute the Serum. Hemolysis.

	NaCl 8.5 per 1000		Ringer (without Bi-(Carbonate)		Tyrode (Carbonated)		Tyrode (Air-treated)		Normosal (Carbonated)	
Vol. Serum diluted to 1/40	0.1	0.2	0.1	0.2	0.1	0.2	0.1	0.2	0.1	0.2
Vol. Saline Sol. Added	0.9	0.8	0.9	0.8	0.9	0.8	0.9	0.8	0.9	0.8
Constant Vol. Susp. of Sheep Globs. 5% (Phys. Sol. NaCl)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Hemolysis after 30 min. at 38°	0	Very Slight	0	Very Slight	Slight	Complete	Very Slight	Strong	Slight	Complete

We employed: (1) Fresh guinea-pig serum; (2) red blood corpuscles (sheep) washed and diluted with NaCl sol., 8.5 per 1000; (3) rabbit anti-sheep serum.
Sensitization: 1 drop of this serum in 10 cc of a suspension of red blood-corpuscles (sheep) at 5 per cent.

to give up treating heated serum with carbonic gas, as an excess of the latter prevents hemolysis. The alexinic activity of the serum of a given guinea pig was, therefore, measured as a function of the different saline solutions used to dilute it. The hydrogen ion concentration of these saline solutions varied according to their content in bicarbonate and in CO_2.

Seelich found that there is no trace of hemolysis in a serum which has been heated for ten minutes at 56° if it has been diluted with a freshly prepared NaCl isotonic solution at 8.5 per cent, whereas when diluted with a solution of Tyrode (carbonated) or Normosal[2] a slight hemolysis can still be observed (Table 28).

When a fresh serum is diluted with a 9 per cent NaCl solution or Ringer

[2] Isotonic solution prepared by "Sächsisches Serumwerk, A. G. Dresden," which contains Na, K, Ca and Mg ions as bicarbonate, phosphate and chloride.

solution, its alexinic power is definitely inferior to that of an analogous dilution made with a Tyrode or Normosal solution containing bicarbonate.

A fresh serum diluted with Tyrode solution through which a current of air has passed for 20 minutes and in which the amount of bicarbonate and the concentration of H^+ ions has been decreased by transformation of the bicarbonate into carbonate, undergoes less hemolysis than a serum diluted with an untreated Tyrode solution.

In connection with this subject we must also mention the old observations of L. and P. von Liebermann[3] and A. Lumière,[4] who showed that the alexenic activity of serum separated from venous blood is much stronger than that of arterial blood less rich in CO_2.

If the biological phenomena depending on alexinic activity are compared to the emulsive power of serum, it is found that the same causes bring about changes in the same direction.

(1) Destruction of alexin after heating above 55°.
(2) Decrease of the alexinic activity with time.
(3) Influence of the content in sodium bicarbonate and in CO_2 in the solutions used to dilute fresh serum or serum heated at 56° on the alexinic activity in the direction of an increase of the latter.

(1) Decrease of the ether coefficient after heating at the same temperature.
(2) Decrease of the ether coefficient with time.
(3) Influence of sodium bicarbonate and of carbonic gas on the ether coefficient in the direction of an increase of the latter.

It therefore seems permissible to assume that the emulsifying power of serum depends on the same mechanisms which condition, in part at least, its alexinic activity; and it is evident that this emulsifying power is directly linked to the structural and physico-chemical modifications enumerated in this book. However, we must admit that our ignorance of the chemistry of proteins does not as yet allow us to establish a complete correlation between these phenomena.

Seelich also showed that the modifications brought about by heating around 57° could be indicated by another method, *i.e.*, interfacial tension.

[3] *Arch. für Hyg.*, 62, 318 (1907).
[4] *Compt. Rend. Acad. Sci.*, 1, 1654 (1928).

Chapter 11

Interfacial Tension as a Function of Temperature

The gel formed by the agitation of serum and ether is a kind of emulsion. This "fixation of ether" obeys the quantitative relations which govern the volume of the non-aqueous emulsifiable phase as a function of a given volume of the aqueous phase and of the quantity and quality of the emulsifier.

Now, we know that the emulsive power of serum is reduced by heating at 56°. Considering that interfacial activity is one of the most important factors in the activity of an emulsifier,[1] this fact might be due to a change in the interfacial activity of the serum, brought about by the modifications undergone by the "serum molecule" during heating. It was therefore interesting to measure the interfacial tension between serum and ether as a function of the temperatures at which the serum had been heated. Unfortunately, however, ether could not be used, as its evaporation is so rapid that it is impossible to make measurements lasting any length of time. Therefore, paraffin oil had to be substituted. This evidently modified the conditions of the problem, but in a known direction, for the interfacial activity of proteins depends on the tension at the interface. Adsorption is evidently different, when a relatively small interfacial tension exists, such as that of ether in contact with water (about 18 dynes), from that produced at the interface of water and paraffin oil, where the tension is relatively high (about 32 dynes).

This commonplace observation expresses only one aspect of a well known thermodynamic principle; but though it is of capital importance it has been disregarded in all biological phenomena and in particular in immunological reactions, as will be seen later. Before continuing we would like to clarify this point of view.

It is known that the second fundamental principle of thermodynamics, also called the Carnot-Clausius principle, can be expressed in the following manner: a system tends toward a state of equilibrium corresponding to the minimum of free energy compatible with its total energy. Boltzmann demonstrated that this state was the most probable. This principle led to the Gibbs-Thomson formula.[2] Now, the free energy of a liquid or of a solution

[1] When the formation, and not the stability of an emulsion, is envisaged.

[2] See Lecomte du Noüy, "Surface Equilibria of Biological and Organic Colloids," p. 187 and following.

is measured by the value of its surface tension and of its interfacial tension. If, therefore, there are substances in the solution capable of decreasing the free energy, these substances will concentrate at the interfaces so as to decrease the total energy of the system. But a selection intervenes at this point which, up to the present, has not been taken into account in the explanation of certain phenomena. Indeed, we have just seen that the adsorption of the substances in solution or suspension is controlled by the *value* of the interfacial tension. Positive adsorption will take place only at a surface where the molecules or particles are capable of *decreasing* the tension. Therefore, if for some reason or other the value of the interfacial tension is decreased, adsorption will no longer take place if the decrease is equal to or greater than that which the molecules in solution are capable of determining by their adsorption. On the contrary, if the interfacial tension is increased, bodies which until then remained in solution will concentrate at the interfaces in order to decrease the tension.

Let us take an example. Consider a system composed of two solutions very slightly or not at all miscible. Suppose the interfacial tension has a value of 35 dynes/cm. If the molecules in solution in one of the solvents normally determine a lowering of the tension corresponding to 32 dynes, they will be adsorbed at all the interfaces, including the liquid-liquid interface, the tension of which will be brought down to 32 dynes. But if the value of the tension at the interface is 32 or 30 dynes to begin with, the same molecules will not be adsorbed at the liquid-liquid interface, whereas they will continue to be adsorbed at the liquid-solid interfaces.

It is therefore possible to determine or prevent the adsorption of certain substances at a certain interface simply by modifying the value of the tension in that interface. By increasing interfacial tension one can force certain substances to adsorb, whereas below a certain value, these same substances would stay in the solution. *The distribution of surface-active molecules in the liquid, in other words, the equilibrium between quantity of adsorbed substances and quantity of dispersed substances, is dependent on the interfacial tension, which can thus prevent the adsorption of certain substances at certain points and not at others.* Credit must be given to Dr. Seelich for calling our attention to these facts.

Measurements were made with the du Noüy interfacial tensiometer described elsewhere,[3] but greatly improved in recent years.

The results of the measurements of interfacial tension as a function of time can be summarized in the following manner:[4]

[3] Lecomte du Noüy, *J. Gen. Physiol.*, 7, 625 (1925) and "Surface Equilibria of Biological and Organic Colloids" p. 166 and following (Manufactured by the Central Scientific Co., Chicago).

[4] Seelich, F., *Biochem. Zeitschr.*, 273, 135 (1934)

(1) The initial drop in the dynamic value of interfacial tension is greater for serum heated at 57° than for normal serum (see Fig. 78). This phenomenon, which is of slight amplitude in all cases, is more marked at relatively weak concentrations where the adsorption is retarded.

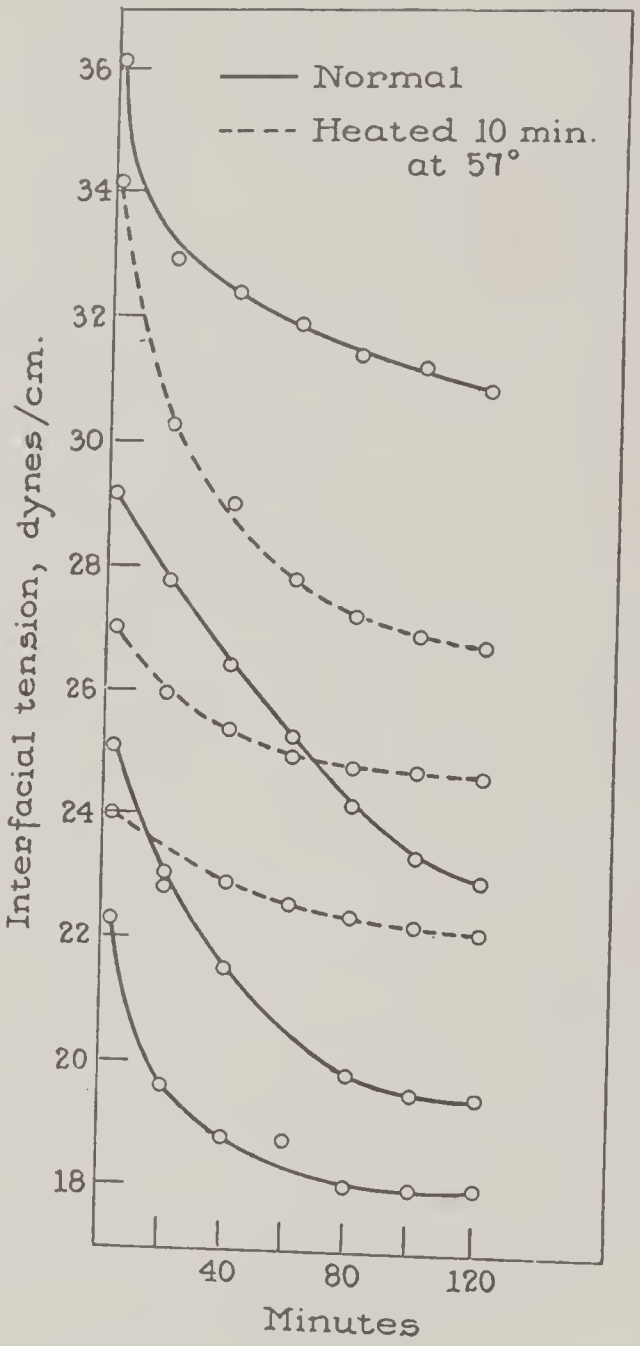

Fig. 78. Horse serum diluted with ringer solution. Interfacial tension against paraffin oil. Measured with the du Noüy Interfacial Tensiometer.

(Solid curve No. 1 —from top—corresponds to serum diluted to 1/1000
No. 2 1/100
No. 3 1/10
No. 4 undiluted)

(2) Normal serum, undiluted or diluted to about 1/100, reaches a static tension which is lower than that of serum heated at 57° in two hours. Serum diluted to more than 1/100 (to 1/1000 for instance) shows, at the end of two hours, an interfacial tension which is much lower than that of pure serum (Fig. 78).

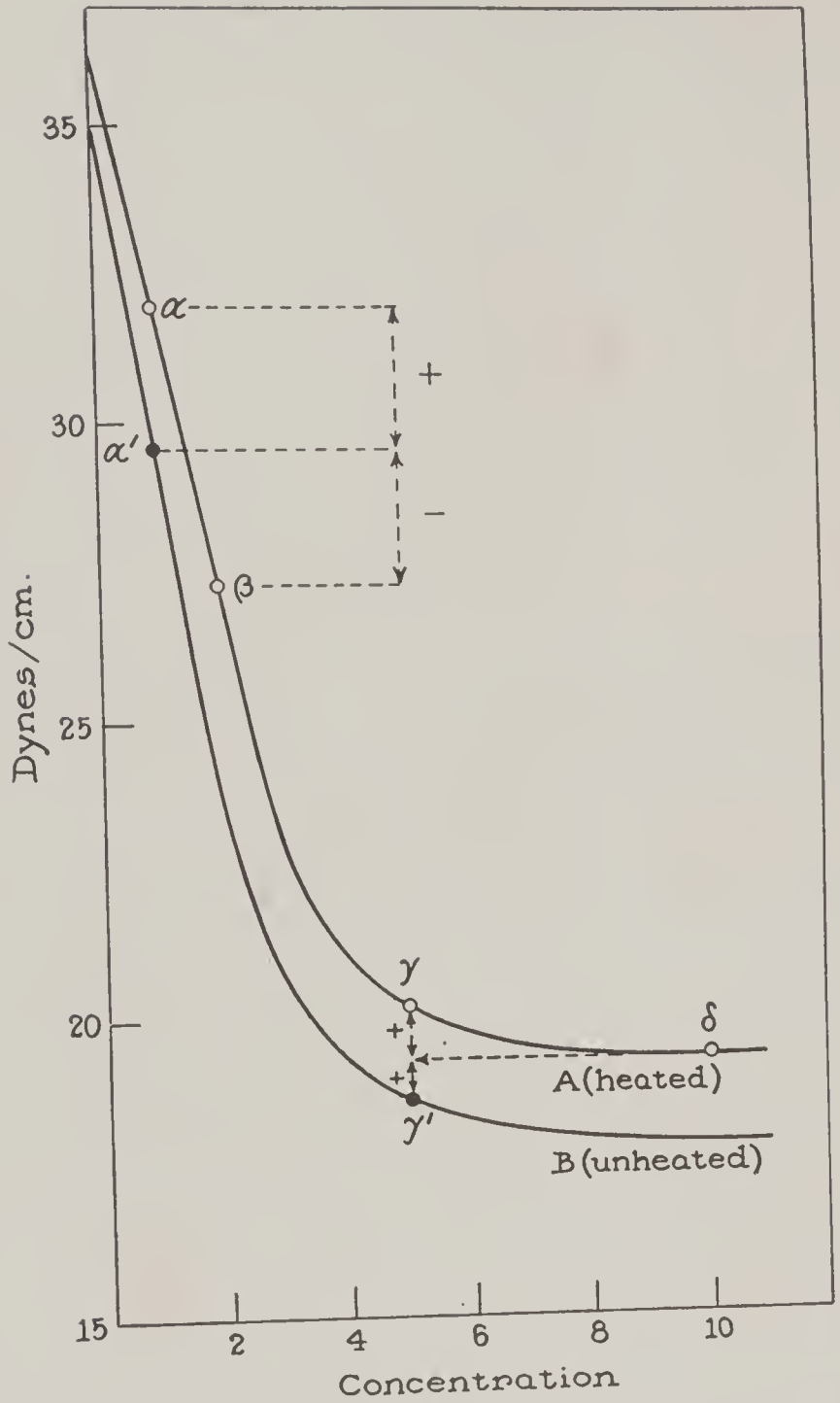

Fig. 79. Schematic chart showing how the surface activity of two solutions can vary in different proportions for the same difference in concentrations, depending on whether they are concentrated or diluted. This difference can go so far as to invert the sign of their relative activities. *See text.*

Seelich explained this curious phenomenon in the following manner. When the surface activity $-\frac{d\gamma}{dc}$ is studied as a function of the concentration of a substance in solution, the curve which represents the variations of the

tension as a function of the concentration has the aspect of the curves in Fig. 79. These curves indicate that the drop is rapid for low concentrations and tends toward a constant value for higher concentrations.[5] The lower curve, B, evidently corresponds to a more active substance than curve A. Now, as the serum molecule is irreversibly hydrated by heating, this should render certain groups more hydrophilic. In other words, after heating, these molecules should have less tendency to adsorb at the surfaces of separation. They are therefore less surface-active. Thus in the case of serum we find ourselves in the presence of two substances (*heated* serum and *unheated* serum) with different activities (with respect to a given interfacial tension), which can be represented by the two curves A and B. But here a new factor intervenes: *the increase of the apparent concentration as a result of heating*, which we demonstrated earlier (see Chaps. 1 and 6). We showed, as the reader may remember, that the serum molecules increased in volume to the detriment of the water (solvent). If the quantity of water decreases with respect to the quantity of substance dissolved, everything takes place as if the concentration had increased. Now it can easily be seen that *at a certain point in the curve* (toward the left) a change in concentration of the heated serum (from $\frac{1}{1}$ to $\frac{2}{1}$ for instance, points α, α', and β) brings about an *inversion* of the value of the surface activity of the heated serum *with respect to the activity* of the unheated serum, α'. At an equal concentration heated serum gives point β instead of point α, as its concentration is *apparently* doubled. Its surface activity is therefore greater in fact, though it has decreased specifically. On the contrary, at another point of the curve (dilution around 1/10) toward the right, a similar apparent increase (of 100 per cent) of the concentration of serum through heating will give point δ instead of point γ and these two points, δ and γ are both *above* the ordinate corresponding to the surface activity of point γ'. Between the two extremes one should find a concentration such that heating would determine an activity exactly equal to that of unheated serum. This hypothesis gives an explanation of the aspect of the curves in Fig. 78 and of the fact that they cross each other, which would be difficult to understand otherwise.[6]

[5] See: Lecomte du Noüy, "Surface Equilibria of Biological and Organic Colloids," pp. 68 and following.

[6] The change of surface tension in heated serum as a result of the *apparent* increase in the concentration of "serum molecules" can be compared to the effect of the addition of inorganic salts or sugars to the solution of a surface-active substance. [Traube, *Ber. d. Deutsch. Chem. Ges.*, **20**, 2644 (1887); Worly, *J. Chem. Soc.*, **105**, 263 (1914); Berczeller, *Bioch. Zeitschr.*, **66**, 173 (1914)]. The surface tension of such a solution is decreased by salts, which increase the surface tension of water, and by sugars, which have practically no influence on the tension of water. The salts and sugars are hydrated, and as a result there is an *apparent* increase in the concentration of the surface-active substance due to the decrease of the quantity of water available. The greater the hydration of the ions the greater is the influence of the salts; and we thus find, once again, the lyotropic series just as in the similar case of the decrease of solubility of slightly

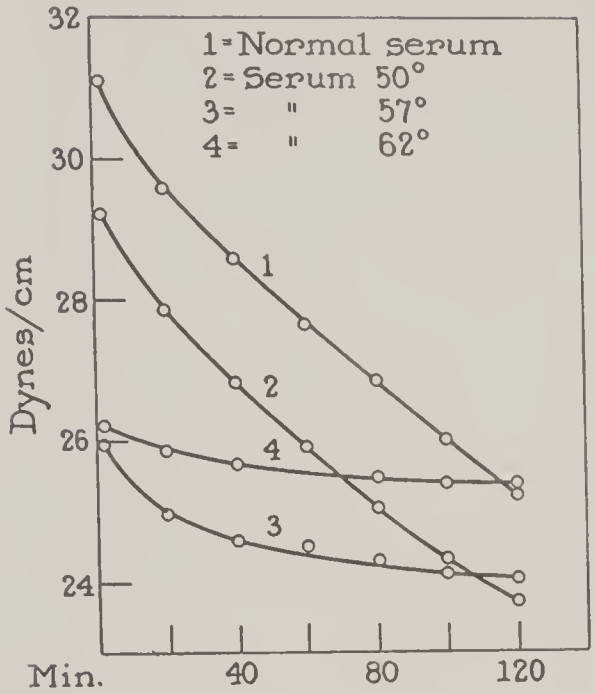

Fig. 80. Interfacial tension against paraffin oil. Horse serum diluted to 1/100 with ringer solution and heated for 10 minutes.

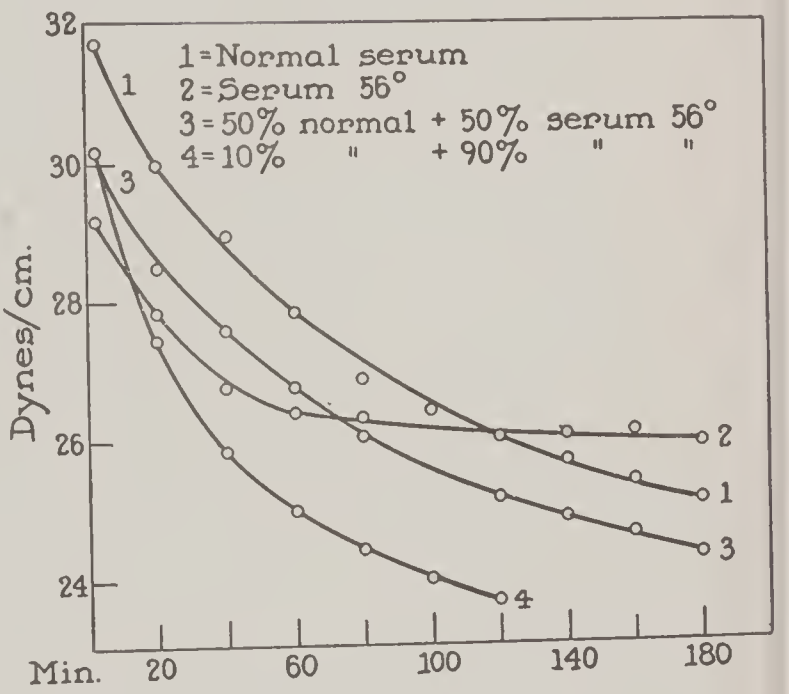

Fig. 81. Interfacial tension against paraffin oil. Horse serum diluted to 1/200 with ringer solution and heated 10 minutes at 56° C.

(3) In the case of serum heated for ten minutes at 62° the static tension, after a given time, seems to be a little higher than in the case of serum heated at 57° (the measurements being made under the same conditions, see Fig. 80). This fact might be due to the increase in viscosity of serum at 62°, which would retard the adsorption.

soluble substances when salts have been added. [Seith, *Zeitschr. Physik. Chemie*, 117, 257 (1925); Freundlich and Schnell, *Zeitschr. Physik. Chemie*, 133, 151 (1928)]. The serum molecules which have increased their hydration and their volume as a result of heating are, by this very fact, in a higher state of concentration with respect to the available water.

(4) The addition of a small quantity of normal serum to serum heated at 56° modifies the curve of interfacial tension as a function of time *in the direction of the curve of normal serum.* The curve of serum heated at 56° for ten minutes and treated in this manner corresponds to a static tension considerably lower than that of serum heated at 56° and to which fresh serum has not been added (see Fig. 81). The addition of 1 per cent of normal serum suffices to modify the curve of serum at 56° in the direction indicated (see Fig. 82).

(5) It was furthermore observed that serum heated for 10 minutes at 57° and left at room temperature for 24 hours, or heated in an incubator for 2½ hours at 37° is modified in such a way that the curve expressing the lower-

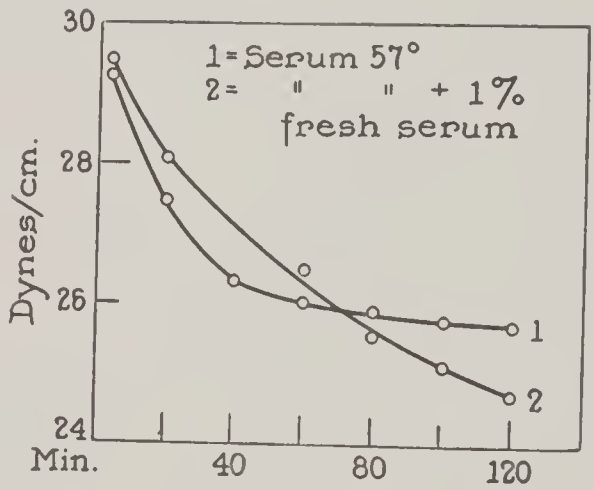

Fig. 82. Interfacial tension against paraffin oil. Guinea-pig serum diluted to ½₀₀ with ringer solution, heated 10 minutes at 57° C. Curve N°2 expresses the behavior of the phenomenon after the addition of 1 per cent of unheated and undiluted serum.

ing of interfacial tension as a function of time corresponds to a static tension which is lower and therefore resembles more closely the curve of unheated serum (see Fig. 83). We found a paper by Gramenitzky[7] which describes the same phenomenon as far as the complement is concerned. Under the same conditions he observed a partial regeneration of the alexinic function.

It is probable that the difference between heated serum and unheated serum would be clearer if the interfacial activity were measured at a lower interfacial tension. The fact that normal serum can bring the interfacial tension down to a point below that of the static tension of heated serum may be due to the presence in normal serum of lipo-proteic complexes which have a greater interfacial activity. It might be possible to find an interface having a value such that these complexes alone could lower it, whereas the serum molecules highly hydrated by heating at 57° would no longer be adsorbed.

[7] Gramenitzky, M., *Bioch. Zeitschr.*, 38, 501 (1912).

It is possible to get an idea of the interfaeial tension at the surface of separation between red blood corpuseles and a physiological solution. This tension should be very slight. From what we said above on the subject of the aetion of the value of interfaeial tension, the presence in normal serum of eomplexes of this kind (lipo-proteins) would suffice to explain the hemolytie funetion in normal serum.

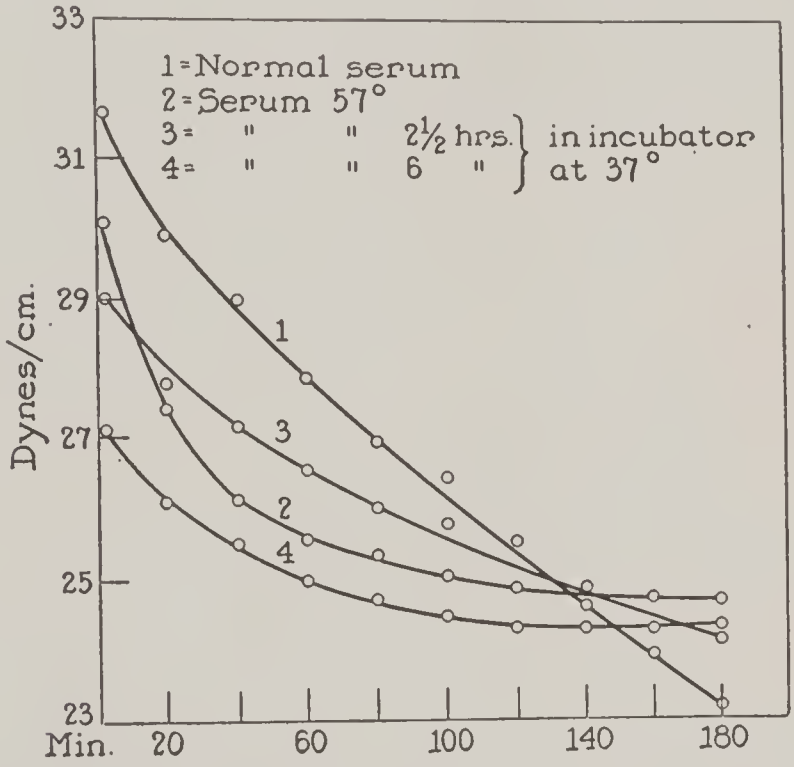

Fig. 83. Interfacial tension against paraffin oil. Guinea-pig serum diluted to 1/200 with ringer solution heated 10 minutes at 57° C. Curve N°3 corresponds to a serum which was kept for 2½ days in the incubator at 37° C. Curve N°4 corresponds to the same serum kept for 6 hours in the incubator at 37° C.

The study of these problems has only just begun, and if these methods are systematieally employed they will undoubtedly give interesting results. By their use it is possible, as the reader has seen, to discover new relations and unknown phenomena which progressively restrict the ground which will have to be eovered before a complete explanation can be given of the physico-chemical mechanisms involved in all immunological reactions. Due to the faet that they were not employed sooner, many years have been lost during which we contented ourselves with verbal entities, the greatest fault of whieh was not that they were hypothetical, but that, by their apparent simplieity, they ereated an artificial state of satisfaction, put euriosity to sleep, and paralyzed research.

Chapter 12

The Ultraviolet Absorption of Serum as a Function of Temperature

A pure substance is generally well defined by its absorption spectrum. The beautiful experiments of Victor Henri and his collaborators[1] showed the important conclusions that could be drawn from curves plotted as a function of the wave length, when the chemical constitution of the body is known. When it is not well known, or not known at all, the conclusions are far from satisfactory. In that case one must be content with the accumulation of data which can perhaps be utilized later. This is the case with blood-serum. Its absorption curve has already been established by different authors,[2] but they did not push the matter further. In order to simplify the problem they generally drew the absorption curves of the albumin and globulins separately. This did not contribute to clarify the question, as these separate elements are not much better known structurally than the hypothetic "serum molecule" as a whole. Nowadays, the latter is sometimes called a "protido-lipidic complex," and its existence is beginning to be admitted by biochemists.

Nevertheless, we thought it might be of interest to make a series of measurements with fresh, normal serum and serum heated at temperatures ranging between 50° and 66° for ten minutes, to ascertain whether the important modifications previously pointed out and starting around 55° did not also bring about variations in the absorption curve.

It is known that the characteristic absorption curve of substances in solution expresses the relative absorption of light, visible or not, by those substances, as a function of the wave length, with respect to the absorption of the solvent for the same wave length. In other words, each point on the curve represents the *ratio* between the absorption of the solution and the absorption of the pure solvent (in our case, water) for a monochromatic radiation.

To obtain this curve the light is dispersed by means of a prism or grating,

[1] Victor Henri, "Études de Photochimie," Paris, Gauthier-Villars, 1919.

[2] Judd Lewis, S., *Proc. Roy. Soc.*, 93, 178 (1922); Stenstrom and Reinhard, *J. Biol. Chem.*, 66, 819 (1925); Smith, F. C., *Proc. Roy. Soc.*, 104, 198 (1929).

after it has passed through the solution, or through the solvent. The two spectra are then photographed side by side and their degree of blackening for the same wave length is compared. Several methods exist by which the optical density of the solution with respect to that of the solvent can be practically estimated. That which gave us the easiest and best results consists in interposing the solvent (water) and the solution (serum) successively on the light path. The thickness is varied but is always identical for the solution and the solvent, and only the time of exposure differs. For instance, in order to obtain the spectrum of the *solvent*, the time of exposure, no matter what the thickness of the liquid, will be five seconds, whereas to obtain the spectrum of the *solution* studied, the time of exposure will vary between six and thirty seconds. This method was advocated by Victor Henri and Wurmser in 1919. On account of the phenomena which take place in the photographic emulsion it is advisable not to go beyond the ratio 6 or 7 for the times of exposure. When this time is insufficient because of the strong absorption of the solvent for certain radiations, it is preferable to decrease the thickness of the liquids in the same proportion, so that the ratio of the times of exposure should remain less than 7.

Assuming that, within the limits of the time exposures employed, the law of Schwarzschild is verified, namely that

$$\frac{I_0}{I} = \left(\frac{T}{t}\right)^n$$

(n being an exponent which varies, according to the photographic emulsion, between 0.9 and 1.0), one can write:

$$\delta = n \log \frac{T}{t}$$

δ representing the optical density of the absorbent for the radiation considered.

Without changing the shape of the curve which represents the variation of δ as a function of the wave length λ, or the position of the absorption bands, it is permissible to assume that $n = 1$.[3] By making t constantly equal to 5 seconds, the following simple formula is obtained:

$$\delta = \log \frac{T}{5}$$

[3] Indeed, the photographic emulsion of our plates receives on one hand a monochromatic light E_0, which reacts on it during a time t, and on the other hand, a light of the same nature, E, which reacts on it during a time T.

It is assumed that the two impressions produce the same blackening. Schwartzschild's law is expressed by the relation:

$$E_0 t^n = E T^n \text{ or } \frac{E_0}{E} = \left(\frac{T}{t}\right)^n$$

the exponent n, varying, according to the emulsions, from 0.9 to 1 in most cases.

This formula was used to establish the curves found in the present chapter. Therefore, in a general case, and for *luminous fluxes* F_0 and F:

$$\frac{F_0}{F} = \left(\frac{T}{t}\right)^n$$

or, when using the logarithms and supposing t constant and equal to 5 seconds, the optical density is equal to

$$\delta = \log \frac{F_0}{F} = n \log \frac{T}{5}$$

Therefore, it is *necessary* to take into account the exponent n, if one wishes to determine the *absolute* values of the optical densities. But if, as in our case, one proposes to determine only the shape of the absorption curve, that is, the variation of δ as a function of the wave length λ, it is no longer necessary to know the value of n. This value must be assumed to be independent of λ.

Indeed, a change in the value of n is equivalent to multiplying the ordinates δ of the absorption curve by the same factor or to changing the scale of ordinates, which evidently does not modify the shape of the curve or the place of the maxima and minima.

As it has been decided not to use a ratio of time exposures higher than 6 (*i.e.*, to limit the exposure of the serum to thirty seconds), $\log \frac{T}{5}$ or δ will always be less than 0.77. As the densities are proportional to the thicknesses, the thickness of the liquid has only to be decreased in order to measure higher densities. If it is decreased by half, for example, the value of δ *expressed with respect to the initial thickness* will be doubled. We shall see a little further on how these magnitudes are combined in order to build up a complete absorption curve.

We employed the ultraviolet spectrophotometer constructed by Jobin & Yvon (quartz spectrometer), which can be used for many purposes owing to its polarization duplicator. We did not take advantage of this device for the spectra published in this chapter. The cells manufactured by the same

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If $n = 1$, the reciprocity law is said to be valid; we then have:

$$\frac{E_0}{E} = \frac{T}{t}$$

In this case, but in this case only, the quantity of light is inversely proportional to the time of exposure necessary to bring about the same blackening of the plate.

In our spectrophotometric technique the monochromatic illumination E_0 and E is proportional to the luminous fluxes F_0 and F of the same wave length which pass through the solvent on one hand and through the absorbent on the other.

firm were extremely accurate and practical and it was possible to vary the thickness of the liquid through which the light passed, from 0 to 10 mm, by 0.01 mm by means of a large graduated drum. We employed ordinary, fine-grain photographic plates (Lumière, Jougla, Guilleminot) 9 x 24 cm, on which 22 spectra could be recorded.

The weak point of these methods is always the difficulty in determining the equality of the blackening on the plates when this is done by the eye alone, without the help of an optical set-up, or of an electrical recording instrument. We therefore used the Jobin & Yvon *Equidensimeter* which afforded the utmost precision possible. This apparatus explores the two contiguous spectra with two thin and identical luminous beams, the dimensions and intensity of which can be regulated. After passing through the photographic plate, these beams are caught by an optical system, and amplified in size so that the two large luminous and contiguous half-fields thus formed can be easily compared. When an identity has been found, the wave length, which has been directly photographed on the plate, can be observed through another ocular piece. The plate is carried on a sliding carriage, which is moved longitudinally by means of a micrometric screw; and as the two telescopes are also carried on a sliding carriage, which can be moved perpendicularly to the sliding frame supporting the plate, it is possible to explore the whole plate with great facility and precision. Under these conditions the position of the region of equal blackening can be determined and expressed in wave lengths to within $\pm 0.001\mu$, or 10 Angstroms.

Technique

After having tried many other luminous sources we finally adopted the Chalonge hydrogen lamp built by S.C.A.D. working under 2000 volts, supplied by a transformer. This lamp gives an absolutely continuous spectrum in the ultraviolet, up to 2,200 A. All the curves published in this chapter were obtained with this source. Experiments showed that the region which interested us could be investigated only when the thickness of the liquid was not more than 0.40 mm and preferably between 0.20 and 0.05 mm. The cell containing water was, therefore, first exposed under a thickness of 0.20 mm, for instance. As mentioned above, the time of exposure for water was always five seconds unless otherwise stated. It was possible to obtain the two spectra, that of water and that of serum, rigorously juxtaposed by means of a mobile screen. The cell containing the same thickness of serum was then put in place and exposed for six or seven seconds.

We quickly found that the most important source of imprecision was the time of exposure. When the exposure was regulated by hand and the time measured by means of a stop-watch giving the fiftieth of a second, the errors frequently attained 1/10th of a second. This was not acceptable.

The points obtained were distributed on either side of the absorption curve and it was impossible to obtain greater precision. What is more, the experiments were extremely tiring because of the sustained attention they required.

It was, therefore, necessary to construct an apparatus which would give the time of exposure automatically from 1 to 30 seconds within 1/50th of a second, at least. The metronome was naturally eliminated as it was not sufficiently accurate. In order to avoid the influence of the temperature of the body of the operator, of the vibrations of the floor, of dust, etc., we resorted to an electrically driven pendulum, the oscillations of which were controlled by an electric clock fed by a single cell and placed, at a distance, on a solid, vibration-free wall. We did not use a synchronous motor connected to the alternating current, as the tolerated fluctuations of the order of 1 to 2 per cent in the periods were too great. The "Magneta" clock which we employed gave us complete satisfaction. When the room temperature was constant within 1 degree ($\pm$ 0.5) the differences during an experiment lasting one hour did not exceed 1/100th of a second, on the average.

The synchronized pendulum was arranged in such a way that the time of exposure could be varied from 1 to 30 seconds by simply moving an index on the dial. It controlled electrically, by means of a relay, an electromagnetic shutter placed in front of the slit. The first series of experiments made with this set-up showed that all the experimental points were on the curve. Thanks to this arrangement it was possible to photograph 18,000 spectra in the ultraviolet.[4]

Calculation of Densities and Plotting of the Absorption Curves

This method enables one to determine in advance the times of exposure necessary to obtain the desired point of the ordinates. Let us suppose that, to begin with, we obtained experimentally and by proceeding tentatively, three points of equal density at the wave lengths: λ = 2,900, 2,530 and 2,470 A. The fact that there are three equalities in these two contiguous spectra indicates that the curve must have a maximum and a minimum. If this result has been obtained with a thickness of 0.2 mm and exposures of 5 seconds for water and 26 seconds for serum, the density δ, which must be plotted as ordinates, will be equal to $\log \frac{T}{t}$ (where t indicates the constant time of exposure of water—5 seconds—and T the time of exposure of serum) or $\log \frac{26}{5} = 0.716$. These points are plotted on a millimeter paper on which the wave lengths are given as abscissae between 2,200 and 3,000 A, for instance. Therefore, there are points on the curve above and below 0.716. If our scale of ordinates covers the range from 0 to 2.00, which is generally

[4] Lecomte du Noüy, P. and M., *Ann. Inst. Pasteur*, 49, 762 (1932).

sufficient, ($\delta = 2.0$, indicating an absorption 100 times greater than that of the liquid of comparison) we must first find the points around $\delta = 0.1$; 0.5; 1.0; 1.5; 2.0, for instance. As we are limited by the ratio $\frac{T}{5}$, which cannot be more than 6 for the reasons given above, we shall be obliged to vary the

Table 29. To Be Used for Calculation of the Absorption Curve in the Ultraviolet when Using the Method of Variable Time Exposures.

Time Exposure of Serum (seconds)	$\delta = \text{Log}\frac{T}{5} \times \frac{0.2}{e}$			
	Thickness 0.4 mm	Thickness 0.2 mm	Thickness 0.1 mm	Thickness 0.05 mm
5				
6	0.0395	0.079		
7	0.058	0.114		
8	0.102	0.204		
9	0.127	0.225		
10	0.150	0.301		
11	0.171	0.342		
12	0.190	0.380	0.760	
13	0.207	0.414	0.828	1.656
14	0.223	0.447	0.894	1.788
15	0.232	0.477	0.954	1.908
16	0.252	0.505	1.010	2.020
17	0.265	0.531	1.062	
18	0.278	0.556	1.102	
19	0.289	0.579	1.158	
20	0.301	0.602	1.204	
21		0.623	1.246	
22		0.643	1.286	
23		0.662	1.324	
24		0.681	1.362	
25		0.698	1.396	
26		0.716	1.432	
27		0.723	1.446	
28		0.748	1.496	
29		0.763	1.526	
30		0.778	1.556	
35		0.845		
40		0.903		
41		0.916		
42		0.925		

thickness of the liquid. (Indeed, our points at $\delta = 0.716$ correspond to log $\frac{26}{5}$, and we can hardly increase T). So as to save time, we established a table with two entries giving the times of exposures as ordinates from 5 seconds to 42 seconds and in four vertical columns, each corresponding to a given thickness (for instance: 0.4; 0.2; 0.1 and 0.05 mm), the values of log $\frac{T}{5}$.

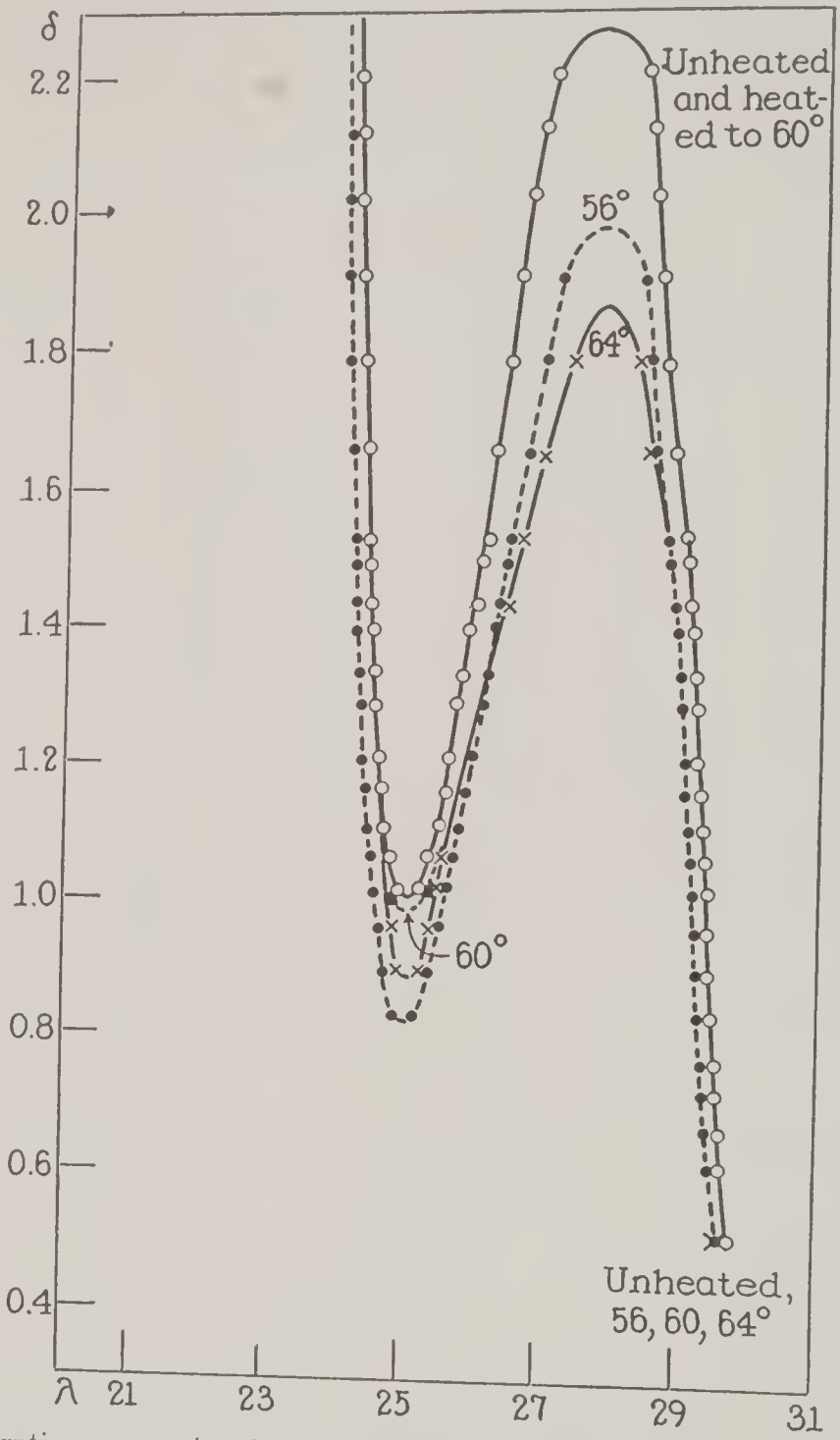

Fig. 84. Absorption curve in the ultra-violet of an immunized (diphtheria) horse serum unheated and heated at different temperatures for 10 minutes. It is seen that in this experiment the curves for 56° and 64° C. are below those corresponding to unheated serum and serum heated at 60° C. Each point (white disks, solid dots or crosses) corresponds to a reading on the Equidensimeter. Each curve represents 25 spectra of serum and 25 spectra of the solvent (water). The same observation holds true for the following figures. ($\lambda \times 100 =$ Angstroms).

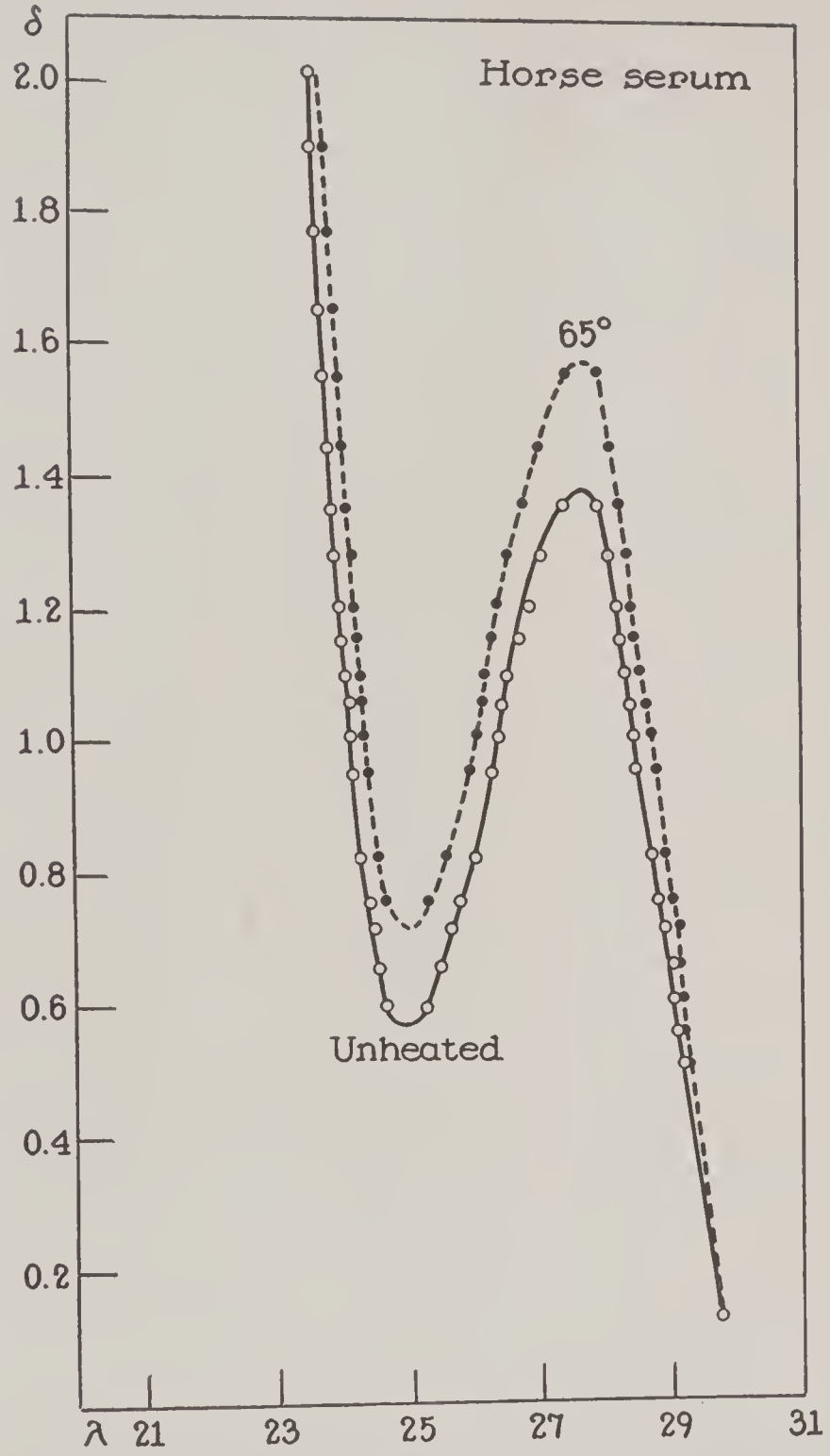

Fig. 85. Absorption curve of horse serum unheated and heated at 65° C. for 10 minutes.

These values vary between 0.0395, (column 0.4 mm, T = six seconds) and 2.020, (column 0.5 mm, T = sixteen seconds). This table is easy to establish if one chooses thickness with a ratio of 1/2, as we have done. (Table 29).

It can be seen that for the same time of exposure a thickness which has

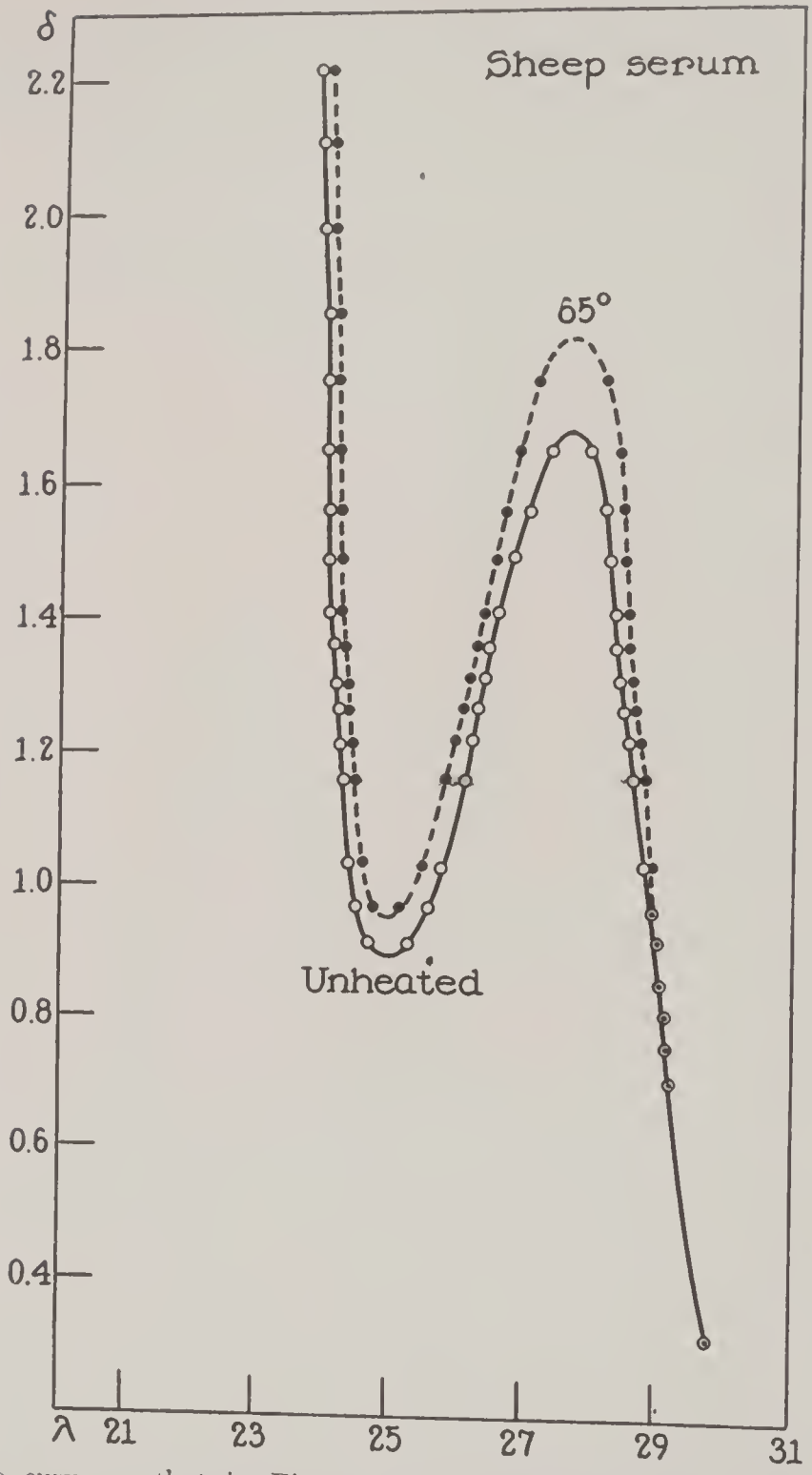

Fig. 86. Same curve as that in Fig. 85, but obtained with sheep serum. Human serum gives absolutely analogous results but the amplitude of the curve is generally decreased.

been doubled corresponds to a density two times lower. For instance, if for a thickness of 0.2 mm and a time of exposure of eighteen seconds, $\delta = 0.556$, $\left(\log \frac{18}{5}\right)$, by reducing the thickness to 0.1, $\delta = 2 \times 0.556 = 1.102$.

Under these conditions any values of δ, if they exist, can easily be obtained, and the most important times of exposure can be decided on in advance. Supplementary spectra can always be made in the spectral region which needs further investigation.

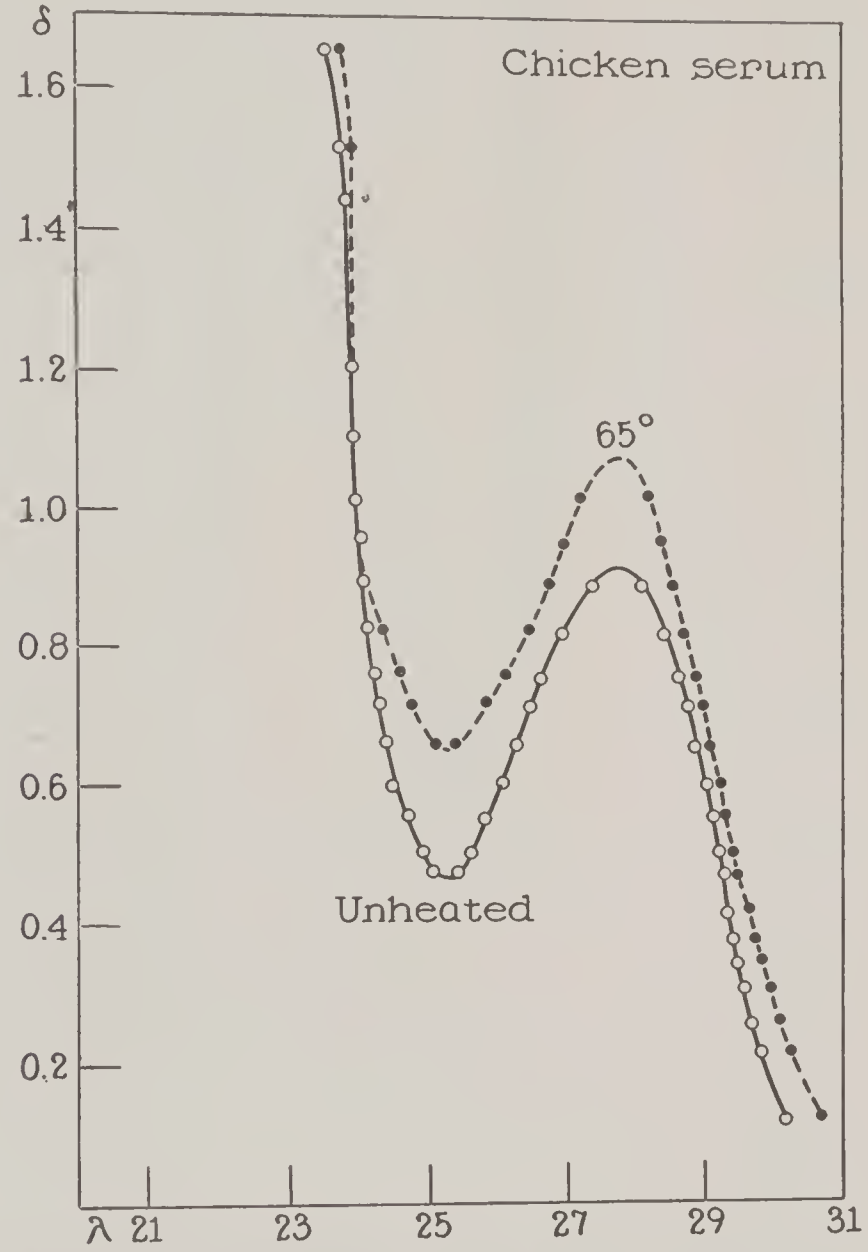

Fig. 87. Same curve but obtained with chicken serum (Class of Birds). A difference in amplitude is *always* observed between the Class of Mammals and the Class of Birds. The dotted curve corresponds to the serum heated at 65° C.

Results

We shall publish only a few absorption curves. The results can be recapitulated in the following manner and are illustrated by Figures 84 to 88.

(1) Normal mammal serum gives a characteristic curve in the ultraviolet showing a maximum and a minimum, the position of which is remark-

ably constant within ± 5 A. The density begins to increase very rapidly around 3,000 A. The maximum is at 2,764 A. ± 5 A.

The curve reaches its minimum at 2,495 A ± 5 A, and goes up almost vertically. The density is considerable at 2,400 A and still increases below 2,300 A.

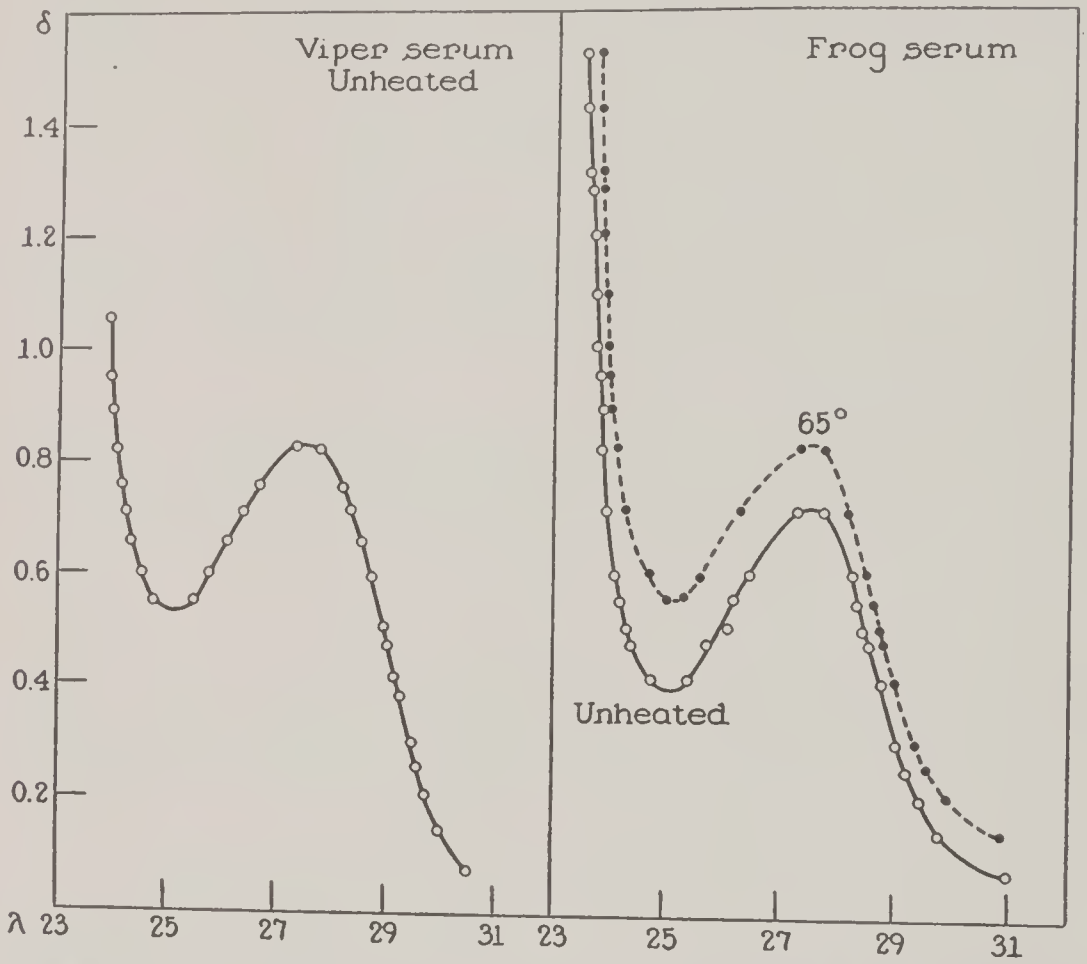

Fig. 88. Same curves: Viper and Frog sera (Class of Reptiles and Class of Batrachians). As is the case for the serum of birds, these curves are much flatter than those corresponding to the Class of Mammals.

2. For 10 minutes' heating,[5] we studied successively:

(a) The wave length of the minimum, which does not vary by an amount greater than the experimental error (± 5 A) as long as the temperature does not reach 65°, when it is displaced toward the visible.

[5] We restricted ourselves to this time of heating because we had observed in all our preceding work (see foregoing chapters) that it was sufficient to clearly put in evidence the quantitative phenomena which we wanted to study. The interest of the experiments in ultraviolet absorption, therefore, depended on the possible correlation between the observed phenomena under identical conditions, and the phenomena which had been established before. It was not so much a question of finding curves differing as a consequence of heating as of finding the modifications brought about in the absorption curve by the same treatment which had brought about definite modifications in the ten phenomena studied up till then.

(b) The wave length of the maximum, which at all temperatures oscillates around its mean position without being systematically displaced.

(c) The optical density of the minimum, which generally decreases at 56° and 64°, and increases at 65°.

(d) The optical density of the maximum, which generally decreases at 56° and 64°, and increases at 65°.

(e) The amplitude, namely, the distance between the density of the minimum and the density of the maximum, generally decreases at *all* temperatures, but sometimes increases at 60°.

The phenomena which characterize most clearly a serum heated for ten minutes are then:

At 56°: The stability of the wave length of the minimum and maximum. The decrease of the density of the minimum (70 per cent of the cases), the tendency toward a decrease in the density of the maximum (67 per cent of the cases).

At 60°: The stability of the wave length of the minimum and maximum.

At 64°: The stability of the wave length of the minimum. The maximum oscillates. The decrease of the density of the minimum and of the maximum (71 per cent of the cases). The decrease of the amplitude in 86 per cent of the cases.

At 65°: The displacement of the wave length of the minimum toward the visible in 79 per cent of the cases (mean value: 11.5 A). The mean stability of the maximum (oscillations). The increase of the density of the minimum (93 per cent of the cases) and of the maximum (79 per cent of the cases).

At 66°: Same characteristics as at 65°, but more marked.

Thus, for the first time we have a clear indication of the *qualitatively* different and deeper perturbation which characterizes heating at 65°, and which results in the so-called "destruction of the sensitizer." This phenomenon is very different from that which takes place at 56° and reaches the chromophoric groups of the molecule.

Analogous results have been obtained not only with the serum of mammals, but with that of birds (hens and cocks). They are much less clear in the case of reptiles (vipers) and batrachians. These experiments gave us the opportunity to observe that the amplitude of the curve in $\sim$ which characterizes serum is always smaller in the case of birds than in the case of mammals.[6] The differences between the classes is less marked, from the point of view of the amplitude of the curve, between the birds, the reptiles and the batrachians. It will be observed that a large number of experimental points were determined for each curve.

[6] Lecomte du Noüy, P. and M., *Compt. Rend. Soc. Biol.*, **116**, 308 (1934).

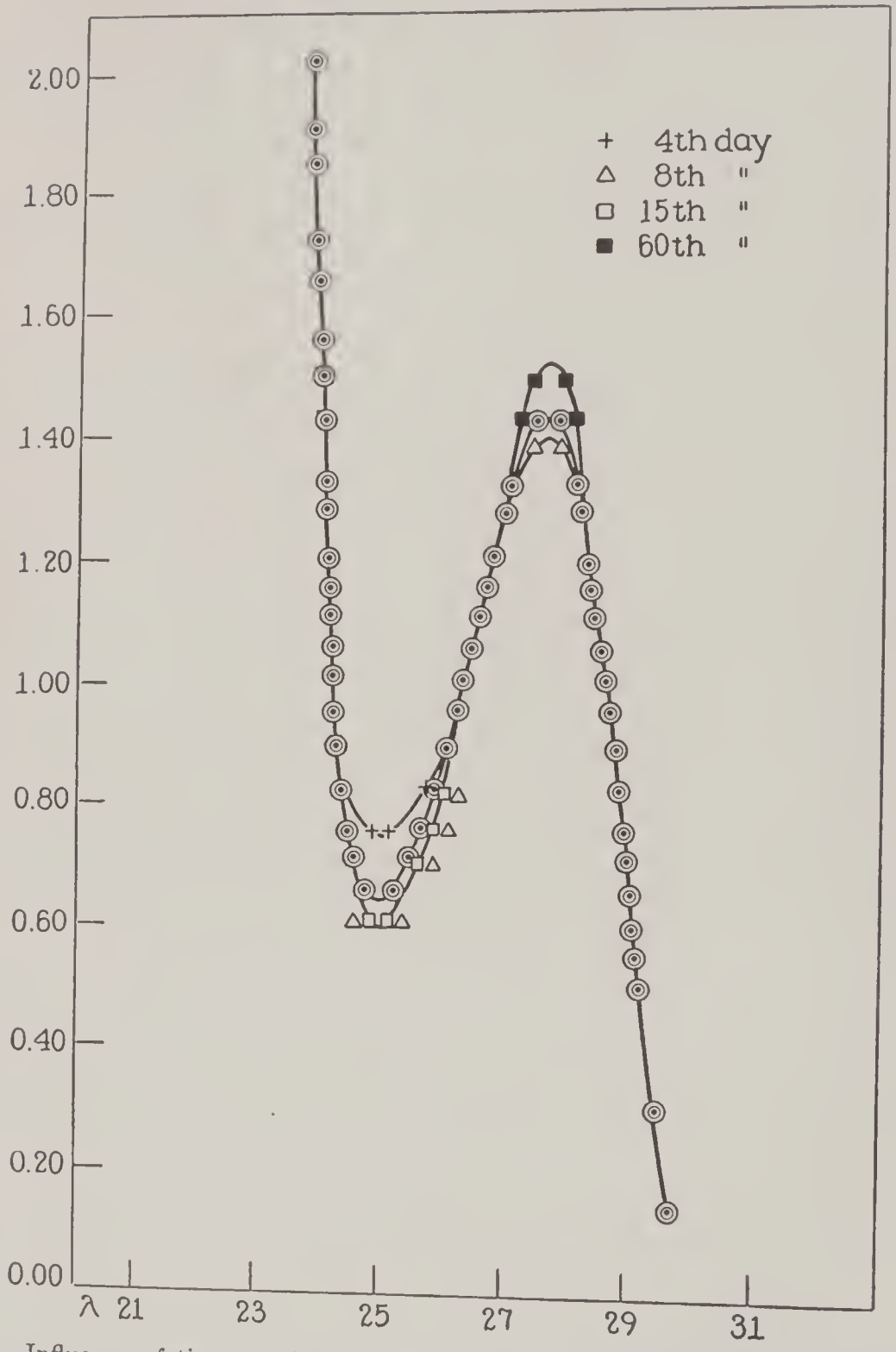

Fig. 89. Influence of time on the absorption spectra of horse serum. The circles correspond to the 2nd day; crosses to the 4th day; triangles to the 8th day; white squares to the 15th day; black squares to the 60th day. The circles correspond to 6 determinations on the 2nd, 4th, 8th, 15th, 30th and 60th day. The abscissae express the wave-length in μ multiplied by 100 and the ordinates express the density δ.

Differences between Serum and Plasma. In the course of these experiments we were able to establish the fact that there were no appreciable differences between the absorption curve of serum and that of the corresponding plasma, as far as the place of the minimum and of the maximum is concerned. Indeed the mean of 6 experiments gives:

Mean value of the place of the minimum: Difference (d) less than experimental error.	Serum: 2495.83 A Plasma: 2495.16 A	$d = 0.67$
Mean value of the place of the maximum: Difference (d) less than experimental error.	Serum: 2764 A Plasma: 2762 A	$d = 2$

For the density of the minimum we obtained (mean of 6 experiments):

Serum: $\delta = 0.741$
Plasma: $\delta = 0.803$ $d = 0.062$

and for the density of the maximum, the same value $\delta = 0.710$ for both the serum and the plasma.

Effect of Age on Serum. Fig. 89 shows that in 60 days the aspect of the absorption curve does not vary systematically. These curves represent the mean of 6 experiments. The curves representing the individual experiments show considerable differences, sometimes in one direction, sometimes in the other.

Absorption Curve of Serum Which Has Been Desiccated and Redissolved. The only marked difference observed was a displacement of the minimum, in all cases, toward the visible region of the spectrum, which could be as high as 50 A. The mean of 6 experiments gives:

Place of the minimum:	Fresh serum: 2496 A Desiccated serum redissolved: 2524 A	$d = 28$

On the other hand, the maximum is much more stable, that is to say, the fluctuations take place in any direction and are always smaller than those of the minimum.

Place of the maximum:	Fresh serum: 2765 A Desiccated serum redissolved: 2767 A	$d = 2$

It must be admitted that, of all the phenomena described and which characterize the critical temperature of serum, these last are the only ones which are not constant in all cases. For profound reasons which we do not know, and which depend on the intimate structure of the protido-lipidic molecules, it sometimes happens, as we have already pointed out, that certain sera behave differently. Nevertheless, a certain number of facts have been clearly demonstrated. In particular, the displacement of the minimum

toward the visible (in 79 per cent of the cases) at 65°, and *only* at that temperature, and the increase of the density of the minimum (93 per cent of the cases) and of the maximum (79 per cent of cases). This fact alone is important, for it indicates the existence of a specific difference in the behavior of serum heated below 65° and at that temperature.

It is, moreover, possible that these phenomena were not observed in 100 per cent of the cases only on account of the brevity of the time of heating. But we have not checked this experimentally.

All the foregoing observations lead to the conclusion that everything happens as if the destruction of the activity of the complement was correlative to the intramolecular hydration which seems to be at the base of all the phenomena revealed by the experiments described in this work. The alexinic activity can therefore be considered in first analysis, either as a structural property of some of the outside chemical groups which are displaced by the penetration of the water molecules, or else as a chemical property destroyed by the fixation of water on the carbonyl groups, for instance.

On the contrary, the specificity of group or of type is a more deep-rooted property which can be altered only by a greater kinetic energy of the water molecules, and which manifests itself by a slight shift of the absorption minimum (from 2,495 to 2,506 A on the average) and by a displacement of the absorption curve as a whole, with respect to the ordinates, toward higher optical densities. In our actual state of ignorance it is impossible to evolve a clear idea of these phenomena as far as the protein molecules are concerned. The key to the problem of the defense of the organism against infectious diseases and of the problem of sensibilization unfortunately lies in the stereochemistry of proteins.

This report of a few results obtained by a science which is still in its infancy suffices to show that even if the benefits are not considerable as yet, nevertheless, no other discipline had been able to obtain them. And this in itself is encouraging.

It is above all important—and we wish to emphasize this point particularly—not to make an error of interpretation and not to believe that, because physical methods are employed, the observed phenomena are of a physical nature. Hemihedrism is a geometrical character, rotatory power is a physical character, but the basic phenomenon is of a chemical, structural nature. It would not be prudent, to say the least, to attribute the selective digestion of the levo-rotatory proteins to a physical property, and yet we were able to define and study this phenomenon only by means of a purely physical method. The same is true of other physical and physico-chemical methods. That is why we have insisted in our preceding works and in this book on the chemical nature of immunological phenomena. It has been

established that eertain physical actions, adsorption, for instance, play a part in the reactions of immunity. But adsorbing power, selective adsorption, and interfacial adsorption are in the end determined by molecular structure, and it seems to us that an arbitrary distinction between two classes of phenomena which are basically the same is more likely to impede than to foster progress, especially when we reach the badly defined and artifieial boundaries between the sciences.

If one is thoroughly eonvineed of this and of the fact that carefully observed faets and their quantitative relation have an unquestionable value, then there is no reason for not resorting to theories and hypotheses, no matter how daring. They eonstitute an essential tool which becomes dangerous only if one gets so attaehed to it that one hesitates to replace it by another, when it is no longer in aecord with the faets.

Conclusion

Irrespective of any hypothesis, it should be possible to draw some conclusions concerning the alexinic activity by taking into account only the experimental facts expounded in this book. These conclusions cannot as yet supply exact data on the chemical nature of the elements on which the alexinic power depends. Nevertheless, they enable one to conceive this property as belonging to the protido-lipoidic complexes, which are molecularly dispersed and which constitute the serum, and not to a separate body. These molecules are made up of elements easily separable, such as albumin, globulins, lipoids, etc. These components are cleavable along surfaces which are not necessarily planes but probably undevelopable surfaces.

We have called attention to twelve distinct phenomena, namely:

(1) Increase of viscosity;
(2) Increase of the rotatory power;
(3) Increase of the rotatory dispersion;
(4) Increase of the absorption of light;
(5) Increase of the scattering of light;
(6) Increase of the depolarization factor;
(7) Increase of electric resistivity;
(8) Increase of the concentration in hydrogen ions;
(9) Decrease of the power of fixation of ether;
(10) Modification of the stability and of the sedimentation of the globulins;
(11) Modification of the interfacial tension;
(12) Modification of the absorption curve in the ultraviolet.

Ten of these phenomena begin around 56° when the serum has been heated for about 10 minutes in a sealed vessel. In undiluted serum, the increase of the concentration of hydrogen ions generally starts around 60°, and the modifications in the absorption curve are clear only at 65°, and do not characterize the "destruction of alexin."

There are, therefore, profound modifications in the structure of the proteins and of the lipo-protidic complex around 55°-56°. These modifications are now a little better known by their influence on ten of the twelve properties which have been studied. On the other hand, we know that this same temperature brings about the destruction of a particular property of serum known as alexin. It is, therefore, logical to admit that the disappearance of this property is related to the structural and chemical modifications deter-

mined by heat in the serum molecule as a whole. The definitive solution will depend on the progress made in the chemistry of proteins and of the protidic complexes.

The hypothesis of an intra-molecular hydration, which we proposed, received an unforeseen confirmation by the experiments of Macheboeuf and Basset, who obtained the coagulation of serum at room temperature by pressure (11,000 to 13,000 atmospheres). By the ultra-pressure method, these authors arrived at the same conclusion as we concerning the existence in the serum of a predominant quantity of "serum molecules" possessing particular immunological properties, which are irremediably destroyed if the albumin is separated from the globulins.

It is through the systematic study of this large, complex molecule and of the globulin fraction of the serum that the solution of all immunological problems will be found.

Other Reinhold Books Relating to this Subject

"Outline of the Amino Acids and Proteins," by Melville Sahyun. 1944. 251 pages. $4.00

"Colloid Chemistry," Volume V, edited by Jerome Alexander. 1944. 1250 pages. $20.00

Volume VI in preparation.

"Proteins, Amino Acids, and Peptides as Ions and Dipolar Ions," by Edwin J. Cohn and John T. Edsall. 1943. A.C.S. Monograph Series. 700 pages. $13.50

"Electrophoresis of Proteins and the Chemistry of Cell Surfaces," by Harold A. Abramson, L. S. Moyer, and M. H. Gorin. 1942. 341 pages. $6.00

"Temperature: Its Measurement and Control in Science and Industry," A Symposium Sponsored by the American Institute of Physics and other scientific and technical organizations. 1941 1375 pages. $11.00

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